December 2004

Environmental Technology Verification Report

IDAHO TECHNOLOGY INC.
R.A.P.I.D.® SYSTEM
FOR THE DETECTION OF
FRANCISELLA TULARENSIS,
YERSINIA PESTIS, BACILLUS ANTHRACIS,
BRUCELLA SUIS, AND ESCHERICHIA COLI

Prepared by
Battelle

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ETV ETV ETV

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Environmental Technology Verification Report

ETV Advanced Monitoring Systems Center

Idaho Technology Inc. R.A.P.I.D.® System for the detection of *Francisella tularensis, Yersinia pestis, Bacillus anthracis, Brucella suis, and Escherichia coli*

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Notice

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development, has financially supported and collaborated in the extramural program described here. This document has been peer reviewed by the Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation by the EPA for use.

Foreword

The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the nation's air, water, and land resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development provides data and science support that can be used to solve environmental problems and to build the scientific knowledge base needed to manage our ecological resources wisely, to understand how pollutants affect our health, and to prevent or reduce environmental risks.

The Environmental Technology Verification (ETV) Program has been established by the EPA to verify the performance characteristics of innovative environmental technology across all media and to report this objective information to permitters, buyers, and users of the technology, thus substantially accelerating the entrance of new environmental technologies into the marketplace. Verification organizations oversee and report verification activities based on testing and quality assurance protocols developed with input from major stakeholders and customer groups associated with the technology area. ETV consists of six verification centers. Information about each of these centers can be found on the Internet at http://www.epa.gov/etv/.

Effective verifications of monitoring technologies are needed to assess environmental quality and to supply cost and performance data to select the most appropriate technology for that assessment. Under a cooperative agreement, Battelle has received EPA funding to plan, coordinate, and conduct such verification tests for "Advanced Monitoring Systems for Air, Water, and Soil" and report the results to the community at large. Information concerning this specific environmental technology area can be found on the Internet at http://www.epa.gov/etv/centers/center1.html.

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List of Abbreviations

AMS	Advanced Monitoring Systems
ASTM	American Society of Testing and Materials
ATEL	AquaTech Environmental Laboratories, Inc.
ATCC	American Type Culture Collection
BSL	Biosafety Level
Ca	calcium
cfu	colony forming unit
cm	centimeter
DI	deionized water
DNA	deoxyribonucleic acid
DW	drinking water
EPA	U.S. Environmental Protection Agency
ETV	Environmental Technology Verification
ID	identification
ITI	Idaho Technology Inc.
L	liter
LCDA	LightCycler Data Analysis
LOD	limit of detection
MB	method blank
Mg	magnesium
mg	milligram
mL	milliliter
MWD	Metropolitan Water District
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PT	performance test
QA	quality assurance
QC	quality control
QMP	Quality Management Plan
R.A.P.I.D.	Ruggedized Advanced Pathogen Identification Device
SOP	standard operating procedure
TSA	technical systems audit

Chapter 1
Background

The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative environmental technologies through performance verification and dissemination of information. The goal of the ETV Program is to further environmental protection by accelerating the acceptance and use of improved and cost-effective technologies. ETV seeks to achieve this goal by providing high-quality, peer-reviewed data on technology performance to those involved in the design, distribution, financing, permitting, purchase, and use of environmental technologies.

ETV works in partnership with recognized testing organizations; with stakeholder groups consisting of buyers, vendor organizations, and permitters; and with the full participation of individual technology developers. The program evaluates the performance of innovative technologies by developing test plans that are responsive to the needs of stakeholders, conducting field or laboratory tests (as appropriate), collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and adequate quality are generated and that the results are defensible.

The EPA's National Exposure Research Laboratory and its verification organization partner, Battelle, operate the Advanced Monitoring Systems (AMS) Center under ETV. The AMS Center recently evaluated the performance of Idaho Technology Inc.'s R.A.P.I.D.® (Ruggedized Advanced Pathogen Identification Device) System for the detection of *Francisella tularensis* (*F. tularensis*), *Yersinia pestis* (*Y. pestis*), *Bacillus anthracis* (*B. anthracis*), *Brucella suis*, and *Escherichia coli* (*E. coli*).

Chapter 2
Technology Description

The objective of the ETV AMS Center is to verify the performance characteristics of environmental monitoring technologies for air, water, and soil. This verification report provides results for the verification testing of Idaho Technology Inc.'s (ITI's) R.A.P.I.D.® System. The following is a description of the R.A.P.I.D.® System based on information provided by the vendor. The information provided below was not subjected to verification in this test.

The R.A.P.I.D.® System is an integrated three-part system for the timely detection and identification of pathogens and biowarfare agents, including anthrax, plague, salmonella, and botulism, in water. The system allows for rapid and specific presumptive identification of threat pathogens in hours rather than days. The system components consist of the ITI 1-2-3 Flow Kit for the purification of deoxyribonucleic acid (DNA), ITI target-specific freeze-dried reagents containing all the necessary ingredients for specific pathogen DNA detection, and the R.A.P.I.D.® 7200 instrument. The operator prepares the DNA from the environmental sample, hydrates the freeze-dried reagents with the DNA sample, runs the R.A.P.I.D.® 7200 instrument, and then reads the auto-analyzed results using the R.A.P.I.D.® software.

The ITI 1-2-3 Flow Kit is a three-step DNA extraction and purification kit that contains the components for purifying DNA from water or other environmental matrix. It removes inhibitors from a sample that would adversely affect a reaction and has been optimized for purifying DNA from difficult-to-process anthrax spores, as well as non-spore forming bacteria. Each kit is optimized and validated for the R.A.P.I.D.® 7200 instrument and contains all the ingredients necessary for DNA purification [one 30 milliliter (mL), one 20 mL, and one 25 mL buffer; 50 bead tubes with lysis buffer; 50 spin filters; 200 receiver tubes; and 50 swabs].

Figure 2-1. Idaho Technology Inc.'s R.A.P.I.D.® 7200 Instrument

The ITI freeze-dried reagents (hybridization probe reagents) are freeze-dried in a single tube and require no refrigeration or freezing. There are multiple gene targets for assay confirmation, with the additional backup of melting curve analysis to confirm any suspected positive test.

The R.A.P.I.D.® 7200 instrument is a field-hardened, air-driven, real-time thermocycler with concurrent fluorescence monitoring that is capable of automatically analyzing samples for the presence of any given DNA sequence. The R.A.P.I.D.® 7200 instrument is based on LightCycler technology and is capable of 45 polymerase chain reaction (PCR) cycles in 30 minutes. It has three color optics, can run on either 110 or 220 volt power, and is watertight in its case. R.A.P.I.D.® software allows the user to automatically collect and interpret data and report results. The software has two user levels, with simple push-button software and auto-analysis or with all the features of the original laboratory instrument for advanced real-time analysis.

The R.A.P.I.D.® 7200 instrument is a 50-pound (22.7-kilogram), portable commercial off-the-shelf system. It operates in various environmental conditions (heat, humidity, salt spray) and has passed a one-meter drop test. The R.A.P.I.D.® instrument includes a backpack, laptop computer, microcentrifuge, and sample capillaries. Its dimensions are 19.4 inches (49.3 centimeters [cm]) by 14.3 inches (36.3 cm) by 10.5 inches (26.7 cm), and its cost is $55,000 U.S., including the centrifuge and laptop. Sample preparation using the ITI 1-2-3 Flow Kit costs approximately $8 per sample, and testing the samples using the freeze-dried reagents costs approximately $17 per sample.

Chapter 3
Test Design and Procedures

3.1 Introduction

The purpose of this verification test of rapid PCR technologies was to evaluate the ability of these technologies to detect the presence of specific bacteria in water and to determine the technologies' performance when specific interferents were added to pure water and when interferents were inherently present in several drinking water matrices. The technologies for this verification test operate based on the PCR process, which involves enzyme-mediated reactions that allow for target DNA (that from the bacteria of interest) replication and amplification through a series of temperature cycles. Before the target DNA can be amplified, however, it must first be extracted from the bacteria and then purified.

Because rapid PCR technologies are anticipated to serve mostly as screening tools in water monitoring scenarios, providing rapid results as to whether or not a pathogen or biological agent is present in the water, this verification test involved only qualitative results. This verification test of the R.A.P.I.D.® System was conducted according to procedures specified in the *Test/QA Plan for Verification of Rapid PCR Technologies*.(1) The performance of the R.A.P.I.D.® System was verified in terms of the following parameters:

- Accuracy
- Specificity
- False positive/negative responses
- Precision
- Interferences
- Other performance factors.

The performance of the R.A.P.I.D.® System was verified by challenging it with various concentration levels of *F. tularensis*, LVS [American Type Culture Collection (ATCC# 29684)] *Y. pestis* CO92, *B. anthracis* Ames strain, *Brucella suis* (ATCC#23444), and *E. coli* O157:H7 (*E. coli*) in American Society of Testing and Materials (ASTM) Type II deionized (DI) water; ASTM Type II DI water spiked with various interferents; and concentrated drinking water (DW) samples obtained from four water utilities from different geographical locations in the United States. Each source of DW represented a unique water treatment process. In addition, the interferent and DW samples were analyzed without adding any contaminant to evaluate the potential for false positive results. The system was only tested for one bacteria at a time.

Contaminant concentrations included the infective/lethal dose concentrations given in Table 3-1 for each contaminant and approximately 2, 5, 10, and 50 times the vendor-reported system limit of detection (LOD). The infective/lethal dose of each contaminant was determined by calculating the concentration at which ingestion of 250 mL of water is likely to cause the death of a 70-kilogram (approximately 154 pounds) person based on human LD_{50} or ID_{50} data.[2] The results from quadruplicate analysis of the contaminant performance test (PT) samples and comparison with the known sample compositions provided information on the accuracy and precision of the R.A.P.I.D.® System. The interferent PT samples contained humic and fulvic acids at two concentrations, both spiked and unspiked with contaminants. Each was analyzed in quadruplicate and provided information on potential matrix interferences.

For the purposes of this test, 1,000 colony forming units per milliliter (cfu/mL) were used to calculate the concentration levels spiked into the PT and DW samples. This vendor-provided concentration level was anticipated to be the level at which quantifiably reproducible positive results could be obtained from a raw water sample using the R.A.P.I.D.® System. This concentration level is referred to as the "system LOD." The system LOD incorporates the sensitivities and uncertainties of the entire R.A.P.I.D.® System, in particular the ITI 1-2-3 Flow Kit DNA purification step, as well as the ITI freeze-dried reagents; and, as such, it is a method detection limit rather than an instrument or reagent-specific detection limit. As mentioned previously, the system LOD provided by the vendor was used specifically as a guideline in calculating sample concentration ranges for use with the R.A.P.I.D.® System in this verification test, and it should be noted that Idaho Technology Inc. does not claim this to be the true LOD of the R.A.P.I.D.® System. Detection limits for individual components of the R.A.P.I.D.® System and the system as a whole may differ and were not verified in this test.

The verification test was conducted at Battelle's Medical Research and Evaluation Facility in West Jefferson, Ohio, as well as Battelle headquarters in Columbus, Ohio, from May 27, 2004, through July 8, 2004. Aqua Tech Environmental Laboratories, Inc. (ATEL) of Marion, Ohio, performed physicochemical characterization for each DW sample, including turbidity, dissolved and total organic carbon, specific conductivity, alkalinity, pH, magnesium (Mg), calcium (Ca), hardness, total organic halides, trihalomethanes, and haloacetic acids. Battelle cultured the bacteria, provided the stock solutions of each bacteria used in this test, and then confirmed the presence and quantity of *F. tularensis, Y. pestis*, *B. anthracis*, *Brucella suis,* and *E. coli* bacteria in the stock solutions using plate enumeration. The stock solutions of *F. tularensis*, *Y. pestis*, *Brucella suis,* and *E. coli* were stored frozen as 1 mL aliquots. The *B. anthracis* stock solutions were refrigerated as 1 mL aliquots. A new 1 mL vial of stock solution was thawed and used for each day of testing. All test samples were prepared from the stock solutions on the day of analysis. All purified DNA was used the same day it was extracted and purified. Each set of replicates for a sample came from the same batch of purified DNA.

Table 3-1. Infective/Lethal Dose of Target Contaminants

Contaminant	Disease Caused by Contaminant	Infective/Lethal Dose Concentration (cfu/mL)
F. tularensis	Tularemia	4×10^5
Y. pestis	Plague	0.28
B. anthracis	Anthrax	200
Brucella suis	Brucellosis	40
E. coli	NA	0.2

NA = not applicable

3.2 Test Samples

Test samples used in this verification test included PT samples, DW samples, and quality control (QC) samples. Each type of test sample, including QC samples, is described further below.

3.2.1 Performance Test Samples

Table 3-2 lists the PT samples analyzed in this verification test for each bacteria. The bacteria were added individually to each spiked sample. PT samples were prepared in ASTM Type II DI water. The first type of PT sample consisted of ASTM Type II DI water spiked at five concentration levels of each individual contaminant. The contaminant PT sample concentrations ranged from the infective/lethal dose concentration to 50 times the vendor-stated system LOD. The infective/lethal dose concentration was analyzed to document the response of the R.A.P.I.D.® System at that important concentration level. Four concentration levels at 2, 5, 10, and 50 times the vendor-reported system LOD, in addition to the infective/lethal dose concentration, were analyzed. Each concentration level for the PT samples was analyzed in quadruplicate.

The second type of PT sample was potential interferent samples. Four replicates of each interferent PT sample were analyzed to determine the performance of the R.A.P.I.D.® System in the presence of humic and fulvic acids. The interferent PT samples contained humic and fulvic acids isolated from Elliot Soil near Joliett, IL, (obtained from the International Humic Substances Society) spiked into ASTM Type II DI water. Each of these interferent mixtures was prepared at two concentration levels. One concentration was near the upper limit of what would be expected in DW [5 milligrams per liter (mg)/L] and one was at a mid-low range of what would be expected (1 mg/L). The 1 mg/L interferent mixture was prepared as 0.5 mg/L humic acid and 0.5 mg/L fulvic acid. Similarly, the 5 mg/L interferent solution was prepared as 2.5 mg/L humic acid and 2.5 mg/L fulvic acid. These interferent levels were confirmed through analysis of aliquots by ATEL. Also, each bacteria was added separately to these samples, along with the potential interferent, at a concentration of 10 times the system LOD and analyzed in quadruplicate.

Table 3-2. Performance Test Samples

Type of PT Sample	Sample Characteristics	Approximate Concentrations (cfu/mL)
Contaminant-only	*F. tularensis*	2×10^3 to 5×10^4
	Y. pestis	0.28 to 5×10^4
	B. anthracis	200 to 5×10^4
	Brucella suis	40 to 5×10^4
	E. coli	0.2 to 5×10^4
Interferent	Contaminants in 0.5 mg/L humic acid and 0.5 mg/L fulvic acid	*F. tularensis*—1×10^4 *Y. pestis*—1×10^4 *B. anthracis*—1×10^4 *Brucella suis*—1×10^4 *E. coli*—1×10^4
	Contaminants in 2.5 mg/L humic acid and 2.5 mg/L fulvic acid	*F. tularensis*—1×10^4 *Y. pestis*—1×10^4 *B. anthracis*—1×10^4 *Brucella suis*—1×10^4 *E. coli*—1×10^4

In all cases, four replicates for each PT sample, DW sample, and QC sample were taken from the extracted and purified product (unspiked) or DNA (spiked) of one sample solution. That is, only one spiked or unspiked sample solution was prepared for each set of replicates and taken through the DNA extraction and purification procedure. Four replicates were then taken from the same purified product or DNA. In an effort to characterize the efficacy of the extraction and purification procedure in the presence of inhibitory substances (humic and fulvic acids), four solutions of humic and fulvic acids at 0.5 mg/L spiked with each contaminant at 10 times the system LOD, were prepared in addition to the samples listed in Table 3-2. Each solution was put through the DNA extraction and purification procedure, and then four replicates from each of the four purified DNA solutions were analyzed on the R.A.P.I.D.® System.

3.2.2 Drinking Water Samples

Table 3-3 lists the DW samples analyzed for each bacteria in this test. Drinking water samples were collected from four geographically distributed municipal sources (Ohio, California, Florida, and New York) to evaluate the performance of the R.A.P.I.D.® System with various sample matrices. These samples varied in their source and treatment and disinfection process. All samples had undergone either chlorination or chloramination prior to receipt. Samples were collected from utility systems with the following treatment and source characteristics:

- Chlorinated filtered surface water
- Chloraminated filtered surface water
- Chlorinated filtered groundwater
- Chlorinated unfiltered surface water.

All samples were collected in pre-cleaned high density polyethylene containers. After sample collection, to characterize the DW matrix, an aliquot of each DW sample was sent to ATEL to determine the following water quality parameters: turbidity, organic carbon, conductivity, alkalinity, pH, Ca, Mg, hardness, total organic halides, concentration of trihalomethanes, and haloacetic acids. The DW samples were dechlorinated with sodium thiosulfate pentahydrate to prevent the degradation of some of the contaminants by chlorine. Because real-world applications of PCR technologies to screen water samples rely on pre-concentration of the water sample to be analyzed, approximately 100 L of each of the above sources of DW were dechlorinated and then concentrated through ultrafiltration techniques to a final volume of 250 mL by the Metropolitan Water District (MWD) of Southern California. As shown in Table 3-3, each DW sample was analyzed without adding any contaminant (i.e., unspiked), as well as after fortification with each individual contaminant at a single concentration level (10 times the vendor-stated system LOD).

Table 3-3. Drinking Water Samples

Drinking Water Sample Description			Approximate Contaminant Concentrations (cfu/mL)				
Water Utility	**Water Treatment**	**Source Type**	***F. tularensis***	***Y. pestis***	***B. anthracis***	***Brucella suis***	***E. coli***
Columbus, Ohio (OH)	chlorinated filtered	surface	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4
MWD of Southern California (CA)	chloraminated filtered	surface	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4
Orlando, Florida (FL)	chlorinated filtered	ground	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4
New York City, New York (NY)	chlorinated unfiltered	surface	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4	unspiked and 1×10^4

3.2.3 Quality Control Samples

QC samples included method blank (MB) samples consisting of ASTM Type II DI water and positive and negative controls, as provided by the vendor. All of the MB QC samples were exposed to sample preparation and analysis procedures identical to the test samples. External positive and negative controls were prepared and used according to the protocol provided by the vendor. At least one positive and negative control were prepared with each batch of samples placed on the R.A.P.I.D.® 7200 instrument. The MB samples were used to confirm negative responses in the absence of any contaminant and to ensure that no sources of contamination

were introduced into handling and analysis procedures. At least 10% of the test samples (eight replicates) for each bacteria and target reagent set were MB samples. The vendor-provided control samples indicated to the technician whether the R.A.P.I.D.® System was functioning properly. If the controls failed for any reason, that batch of samples would be discarded and the extracts reanalyzed. To the extent practicable, the test samples were analyzed blindly by having the technician label the vials with only a sample number prior to the DNA purification step, so that the samples were tracked through the purification, PCR, and detection steps by only a sample number. Due to special facility use, the identity of the target bacteria was always known by the technician.

3.3 Reference Methods

3.3.1 Plate Enumeration

For all contaminants, plate enumeration was used to quantify bacteria to confirm the concentration of the stock solutions of these contaminants. The Battelle standard operating procedure (SOP) followed was SOP No. MREF X-054, *Standard Operating Procedure (SOP) for the Enumeration of BSL-2 and BSL-3 Bacteria Samples Via the Spread Plate Technique.*

Prior to testing, the *F. tularensis*, *Y. pestis*, *Brucella suis*, and *E. coli* were grown and then suspended in phosphate buffered saline (PBS). Twenty-five or more individual 1 mL aliquots of stock solution were prepared from each original PBS stock solution. Three 1mL aliquots were randomly taken for enumeration, while the others were frozen for later use in sample preparation. Each bacteria was enumerated on each of the three selected 1 mL aliquot to confirm the determined concentration.

The *B. anthracis* came from a lot of spores prepared by Battelle and stored in a 1% stock solution of phenol in water. Prior to testing, an aliquot of the *B. anthracis* solution described above was centrifuged, the supernatant consisting of the phenol/water solution was decanted from the spores, and the spores were reconstituted with DI water. This process was repeated two times to ensure that the spores were suspended only in DI water. This DI water suspension of spores was then aliquoted into 1 mL portions as with the *F. tularensis*, *Y. pestis*, *Brucella suis*, and *E. coli*. Because of the known stability of *B. anthracis* spores and based on general facility protocol, the aliquots were refrigerated instead of frozen. An aliquot was enumerated in triplicate prior to testing to confirm the concentration. Another aliquot was enumerated during the verification test to further verify the concentration of *B. anthracis* in the stock solution vials.

3.3.2 Drinking W

Because most of the contaminants tested can occur naturally in water, and because rapid PCR technologies cannot distinguish between live and dead organisms, each unspiked concentrated DW sample was plate enumerated to verify, to the extent practicable, the presence or absence of the contaminant of interest. The samples were plated onto tryptic soy agar plates with 5% sheep blood and incubated at 30 to 35°C. After 20 hours of incubation, the unspiked OH, CA, and NY

DW samples produced lawns of bacteria with a level of contamination estimated to be greater than 1×10^3 cfu/mL. The unspiked FL DW sample showed only 10 to 100 cfu/mL estimated concentration levels after 20 hours. After further incubation, the FL DW sample produced bacteria at a concentration estimated to be greater than 1×10^3 cfu/mL. Each DW sample had at least three distinct types of bacteria growing. Gram stains were performed on any distinct colony types visible in each sample to gain further insight into the colony morphology. For OH and CA DW, three Gram negative bacteria colonies were identified. For NY, four Gram negative colonies were identified; and, for FL, both Gram negative and positive colonies were present.

The CA DW was further evaluated for the presence of *F. tularensis* based on the potential positive results for unspiked CA DW samples during the verification test. An aliquot of the water was plated onto cystine heart agar (*F. tularensis* selective media) and incubated at 30 to 35°C. A single colony type (Gram negative rods) grew on the plates and was subjected to biochemical tests (catalase, oxidase, β-lactamase, and urease) for the presumptive identification of *F. tularensis*. The biochemical test results came back oxidase positive, indicating that the bacteria were not *F. tularensis*. Further identification tests were not conducted on other DW samples because no confirmed positive responses were detected in the remaining unspiked DW samples.

3.4 Test Procedure

3.4.1 Sample Handling

All testing for this verification test was conducted within Battelle laboratories staffed with technicians trained to safely handle *F. tularensis, Y. pestis*, *B. anthracis*, *Brucella suis*, and *E. coli* bacteria. The technician operating the R.A.P.I.D.® System had prior PCR experience. *F. tularensis* and *E. coli* samples were tested in a Biosafety Level 2 (BSL-2) laboratory; while *Y. pestis*, *B. anthracis*, and *Brucella suis* samples were tested in a BSL-3 laboratory. Appropriate safety guidelines associated with each laboratory were followed throughout the verification test. Each day, fresh samples were prepared from a thawed vial of frozen or refrigerated stock solution in either DI water, an interferent matrix, or a DW matrix. Concentration levels for spiked samples at various multiples of the R.A.P.I.D.® System's LOD (2, 5, 10, and 50 times the system LOD for PT samples, and 10 times the system LOD for interferent and DW samples) were calculated from the system LOD provided by the vendor. Sample solutions were prepared to these concentrations based on the concentration of the bacteria stock solution, which was determined through triplicate plate enumeration prior to testing. Each sample was prepared in its own container and labeled only with a sample identification (ID) number that also was recorded in a laboratory record book along with details of the sample preparation. Samples were diluted to the appropriate concentration using volumetric pipettes and glassware. Each sample was prepared in 5 mL quantities.

Despite rigorous sample preparation efforts, solutions consisting of low bacterial concentrations, such as the *Brucella suis* or *Y. pestis* infective/lethal dose, may have no DNA present in a given sample or aliquot.[3,4] The rationale for this is based on the Poisson statistical distribution, where there is some probability that a sample taken will contain no particles (i.e., bacteria or target

DNA) and thus yield a negative result.[3,4] As a practical example, assume that 1 mL contains exactly five particles (i.e., bacteria or target DNA) of interest. If one takes ten 0.1 mL samples and analyzes them, the maximum number of positives will be five out of the ten samples. From this it follows that there will be at least five negatives. Random variation in the sampling will cause this ratio to change. This verification test was not designed to differentiate between the stochastic nature of the low concentration samples and the capabilities of the assays, but this phenomenon should be noted.

3.4.2 Sample Preparation and Analysis

Three steps were carried out to test a liquid sample for the presence of *F. tularensis, Y. pestis*, *B. anthracis*, *Brucella suis,* and *E. coli* bacteria: (1) DNA extraction and purification using the ITI 1-2-3 Flow Kit, (2) PCR setup using the freeze-dried reagents, and (3) PCR and analysis using the R.A.P.I.D.® 7200 instrument and software. To perform these steps, the laboratory work area was separated into three distinct areas: DNA extraction and purification was performed in one area; the PCR setup, involving reconstituting the freeze-dried reagents and loading the capillaries, was performed in a separate area; and loading and running the instrument (the R.A.P.I.D.®) was done in another area. These steps are described below.

First, the DNA was extracted and purified from the sample using the ITI 1-2-3 Flow Kit. The entire 5 mL sample was taken through this purification procedure. The kit instructions specific to processing a water sample were followed. After the purification step was complete, the PCR samples were prepared in another area using the freeze-dried reagents. This process involved reconstituting the freeze-dried reagents. The reagents for each sample and control placed on the R.A.P.I.D.® 7200 instrument come in individually sealed vials labeled as “positive,” “negative,” and “unknown.” First, the negative controls were reconstituted by combining 40 µL of provided sterile PCR grade water with the freeze-dried reagent in the “negative” vial. The reconstituted negative control was then split into two capillaries, which were capped and briefly centrifuged to pull all of the liquid into the bottom of the capillaries. The test samples were prepared next. For the sample preparation, the “unknown” freeze-dried reagent vial specific to the bacteria being tested was used. A volume of 20 µL of purified sample DNA and 20 µL of sterile PCR grade water were combined with the freeze-dried reagents in the vial. As with the negative controls, the sample “unknown” reagent mix was split into two capillaries, capped, and then briefly centrifuged. Each test sample replicate was prepared using an individual pathogen-specific “unknown” freeze-dried reagent vial and then split into two capillaries per the R.A.P.I.D.® protocol. After all of the samples were prepared, the positive control was prepared in the same manner as the negative control, only using the “positive” freeze-dried reagent vial. The controls and “unknown” freeze-dried reagent vials came packaged together for each target.

Once all of the capillaries were filled and capped, they were loaded onto the R.A.P.I.D.® 7200 instrument carousel and the PCR run was started per the instructions provided with the instrument. The carousel can hold up to 32 capillaries, or two split controls (positive and negative) and 14 split samples. For this verification test, to ensure that an entire set of replicates for a sample was run at the same time, three sets of four replicates along with positive and negative controls were generally placed on the carousel for one PCR run. Three sets of four sample replicates and positive and negative controls are considered a batch. The PCR program

was loaded using the Advanced Options "Run" feature (version 1.2.14). After the R.A.P.I.D.® 7200 instrument had completed its PCR program run, which consisted of 45 cycles on the thermal cycler, the results were analyzed using the R.A.P.I.D.® software. For the purposes of this test, the Advanced Options LCDA (LightCycler Data Analysis) (version 3.5.28) quantification function was used to interpret the results. In this data analysis option, the amplification data for each capillary are plotted as fluorescence versus cycle number (from the thermal cycler program). The Second Derivative Maximum method was used to determine a crossing point, or the fractional cycle number where the sample fluorescence is differentiated from the background fluorescence. The baseline adjustment was made by the software using the "Arithmetic" setting, and the noise band (discrimination between noise and actual amplification in the fluorescence curves) was set automatically by the software. The crossing point was generated by the software based on the second derivative maximum value of each amplification curve. The resulting amplification plots (plots of fluorescence versus the thermal cycler cycle number) and crossing point values were used to determine the results for each sample. A sample was considered positive (bacteria detected in the sample) if a crossing point was assigned to that sample and the amplification curve for that sample was above the baseline. A sample was considered negative (no bacteria detected in the sample) when no crossing point value was assigned by the software and the amplification curve was along the baseline with no sign of exponential increase. A sample was also considered negative if a crossing point value was assigned to the sample but no amplification above the baseline was apparent for that sample's amplification curve. Samples were considered positive or negative only if both capillaries from the split analysis of the sample showed positive or negative results. Results for a sample were deemed inconclusive when the two capillaries for the sample did not agree with each other (one was positive and one was negative). The negative controls were considered successful if no amplification was present (and thus no crossing point was assigned to the sample). The positive controls were considered successful if amplification above the baseline was noted on the plot and a crossing point was assigned to the samples. The technician recorded the sample ID number on a sample data sheet along with the qualitative results (positive or negative) for each sample.

For *F. tularensis*, *Y. pestis*, and *B. anthracis*, more than one assay (freeze-dried reagent target) was used for the analysis of each bacteria. In other words, multiple sets of "unknown" and control assays were used to determine the presence or absence of the same bacteria in the PT and DW samples. Each set of assays for a particular bacteria were specific to different gene targets for that bacteria. Three assays were tested for *B. anthracis* (Target 1, Target 2, and Target 3), while two assays were tested for *F. tularensis* and *Y. pestis* (Target 1 and Target 2 for each). In each case where multiple freeze-dried reagent targets were tested for a given bacteria, the replicates for each sample tested for each target came from the same purified sample DNA. For example, when the infective/lethal dose sample for *B. anthracis* was tested, the same batch of purified sample DNA was used to analyze four replicates for Target 1, four replicates for Target 2, and four replicates for Target 3 for the *B. anthracis* reagents.

3.4.3 Drinking Water Characterization

An aliquot of each DW sample, collected as described in Section 3.2.2, was sent to ATEL prior to concentration to determine the following water quality parameters: turbidity; concentration of dissolved and total organic carbon; conductivity; alkalinity; pH; concentration of Ca and Mg;

hardness; and concentration of total organic halides, trihalomethanes, and haloacetic acids. Table 3-4 lists the methods used to characterize the DW samples, as well as the characterization data from the four water samples used in this verification test. Water samples were collected and water quality parameters were measured by ATEL in January 2004. Some of the water quality parameters may have changed slightly prior to verification testing.

Table 3-4. ATEL Water Quality Characterization of Drinking Water Samples

			Sources of Drinking Water Samples			
Parameter	**Unit**	**Method**	**Columbus, Ohio (OH DW)**	**MWD, California (CA DW)**	**Orlando, Florida (FL DW)**	**New York City, New York (NY DW)**
Turbidity	NTU	EPA 180.1[5]	0.2	0.1	0.5	1.3
Dissolved organic carbon	mg/L	SM 5310[6]	1.9	2.3	1.7	1.5
Total organic carbon	mg/L	SM 5310[6]	1.6	2.1	1.8	2.1
Specific conductivity	micro-Siemens	SM 2510[6]	357	740	325	85
Alkalinity	mg/L	SM 2320[6]	55	90	124	4
pH		EPA 150.1[7]	7.33	7.91	7.93	6.80
Ca	mg/L	EPA 200.8[8]	42	35	41	5.7
Mg	mg/L	EPA 200.8[8]	5.9	1.5	8.4	19
Hardness	mg/L	EPA 130.2[7]	125	161	137	28
Total organic halides	µg/L	SM 5320[6]	360	370	370	310
Trihalomethanes	µg/L/analyte	EPA 524.2[9]	26.9	79.7	80.9	38.4
Haloacetic acids	µg/L/analyte	EPA 552.2[10]	23.2	17.6	41.1	40.3

NTU = nephelometric turbidity unit
µg = microgram

Chapter 4
Quality Assurance/Quality Control

Quality assurance/quality control procedures were performed in accordance with the quality management plan (QMP) for the AMS Center[11] and the test/QA plan for this verification test.[1]

4.1 Sample Chain-of Custody Procedures

Sample custody was documented throughout collection, shipping, and analysis of the samples. Sample chain-of-custody procedures were generally those provided in the guidelines in ASAT.II-007, *Standard Operating Procedure for Chain of Custody for Dioxin/Furan Analysis*. The chain-of-custody forms summarized the samples collected and analyses requested and were signed by the person relinquishing samples once that person had verified that the custody forms were accurate. The original sample custody forms accompanied the samples; the shipper kept a copy. Upon receipt at the sample destination, sample custody forms were signed by the person receiving the samples once that person had verified that all samples identified on the custody forms were present in the shipping container.

4.2 Equipment Calibration

The R.A.P.I.D.® System and all associated reagents and supplies specific for the detection of *F. tularensis*, *Y. pestis*, *B. anthracis*, *Brucella suis*, and *E. coli* were provided to Battelle by the vendor. This system required no calibration. The performance of the system was monitored through positive and negative controls. For DW characterization and confirmation of the possible interferent, analytical equipment was calibrated by ATEL according to the procedures specified in the appropriate standard methods. Pipettes used during the verification test were calibrated according to Battelle SOP VI-025, *Operation, Calibration, and Maintaining Fixed and Adjustable Volume Pipettes.*

After completion of the verification test, a Rainin pipette Lite 200 used during most of the testing in the BSL-3 laboratory (for *Y. pestis, B. anthracis,* and *Brucella suis*) was found to be out of calibration. The pipette was found to deliver liquids in excess of the volume specified on the pipette. It is not known if the pipette went out of calibration before, during, or after the verification test. The pipette was used in two steps: sample solution preparation and to deliver liquids to reconstitute the freeze-dried reagents. There is no definitive evidence to indicate that the pipette did or did not affect the results for the BSL-3 bacteria.

4.3 Characterization of Contaminant Stock Solutions

F. tularensis, Y. pestis, *B. anthracis*, *Brucella suis,* and *E. coli* were grown and prepared by Battelle. All bacteria were plate enumerated in triplicate for confirmation of the concentration of the 1 mL aliquot stock solutions. Prior to enumeration, the *B. anthracis*, originally stored as a 1% stock solution of phenol in water, was aliquoted and washed twice with DI water and resuspended in only DI water for analysis.

The lot of *B. anthracis* Ames Strain spores used for this verification test were characterized in September 2003 by Battelle and the Centers for Disease Control and Prevention. This characterization involved evaluation of 11 criteria, including the percent of vegetative cells present, the viable spore count, the guinea pig 10 day LD_{50}, as well as DNA fingerprinting and gene sequencing. This lot of spores met all 11 acceptance criteria, proving that the spores were viable and of the specified strain (Ames). The vegetative cell count indicated that the stock solution of spores was 99.94% pure spores, with only 0.06% of the solution containing vegetative cells.

The Battelle SOP No. MREF X-054, *Standard Operating Procedure (SOP) for the Enumeration of BSL-2 and BSL-3 Bacteria Samples Via the Spread Plate Technique,* was followed for the plate enumeration of *F. tularensis, Y. pestis*, *B. anthracis*, *Brucella suis,* and *E. coli*. The results of the plate enumerations for each bacteria are presented in Table 4-1. For all bacteria, the plate enumeration was conducted prior to testing. Because the *B. anthracis* stock solutions were stored at 2 to 8°C, another 1 mL aliquot stock solution vial was enumerated during testing activities to further confirm the concentration of the stock solutions. The average of triplicate enumerations for each bacteria was used to calculate and prepare all spiked sample solutions. The percent difference between the concentration of the initial preparation of *B. anthracis* spores and the second analysis of these spore during testing was 23%. Because this difference falls within the bounds of expected plate enumeration error and is close to the standard deviations found for the plate enumerations of other bacteria used in this verification test, the concentration determined from the initial set of plate enumerations on the *B. anthracis* spores was used in calculating solution concentrations.

4.4 Quality Control Samples

MB samples consisting of ASTM Type II DI water, and positive and negative control samples, as provided in the R.A.P.I.D.® System, were analyzed to help identify potential cross-contamination issues as well as verify that the PCR process was functioning properly. Positive and negative control samples were run with each set of samples placed on the R.A.P.I.D.® 7200 instrument. Eight MB replicates were analyzed over the course of the verification test for each bacteria (or bacteria assay).

Each set of eight MB sample replicates for all *Y. pestis*, *B. anthracis*, *Brucella suis,* and *E. coli* targets returned negative results. One set of MB replicates for the *F. tularensis* Target 1 returned one positive and two inconclusive results, indicating possible contamination. Other samples on

Table 4-1. Contaminant Triplicate Plate Enumeration Data

Bacteria	Plate 1 Concentration (cfu/mL)	Plate 2 Concentration (cfu/mL)	Plate 3 Concentration (cfu/mL)	Average (cfu/mL)	Relative Standard Deviation
F. tularensis	1.0×10^{9}	1.1×10^{9}	1.2×10^{9}	1.1×10^{9}	9%
Y. pestis	5.8×10^{7}	6.5×10^{7}	5.0×10^{7}	5.8×10^{7}	13%
B. anthracis (initial prep)	8.7×10^{7}	8.1×10^{7}	7.8×10^{7}	8.2×10^{7}	6%
B. anthracis (second analysis)	5.7×10^{7}	5.7×10^{7}	7.6×10^{7}	6.3×10^{7}	17%
Brucella suis	1.5×10^{10}	1.6×10^{10}	1.4×10^{10}	1.5×10^{10}	7%
E. coli	7.3×10^{8}	5.0×10^{8}	9.0×10^{8}	7.1×10^{8}	28%

that day's testing did not show signs of contamination, and the second *F. tularensis* target run from the same purified DNA returned all negative results for the four replicates.

No positive controls failed during the verification test. Three negative controls failed over the entire course of testing. In these instances, the batch of samples was loaded again and rerun using new reagents. For two batches of samples, the carousel was misaligned, producing incomprehensible results. These batches of samples were rerun. The results from all reruns were used in the data analysis.

4.5 Audits

4.5.1 Technical Systems Audit

The Battelle Quality Manager conducted a technical systems audit (TSA) on June 11, 2004, to ensure that the verification test was performed in accordance with the test/QA plan[(1)] and the AMS Center QMP.[(11)] As part of the audit, the Battelle Quality Manager reviewed the standards and methods used, compared actual test procedures to those specified in the test/QA plan, and reviewed data acquisition and handling procedures. Observations and findings from this audit were documented and submitted to the Verification Test Coordinator for response. No findings were documented that required any significant action. The records concerning the TSA are stored for at least seven years with the Battelle Quality Manager.

4.5.2 Audit of Data Quality

At least 10% of the data acquired during the verification test was audited. Battelle's Quality Manager traced the data from the initial acquisition, through reduction and statistical analysis,

to final reporting, to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

4.6 QA/QC Reporting

Each assessment and audit was documented in accordance with Sections 3.3.4 and 3.3.5 of the QMP for the ETV AMS Center.[11] Once the assessment report was prepared, the Verification Test Coordinator ensured that a response was provided for each adverse finding or potential problem and implemented any necessary follow-up corrective action. The Battelle Quality Manager ensured that follow-up corrective action was taken. The results of the TSA were sent to the EPA.

4.7 Data Review

Records generated in the verification test were reviewed before these records were used to calculate, evaluate, or report verification results. Table 4-2 summarizes the types of data recorded. The review was performed by a Battelle technical staff member involved in the verification test, but not the staff member that originally generated the record. The person performing the review added his/her initials and the date to a hard copy of the record being reviewed.

Table 4-2. Data Recording Process

Data to Be Recorded	Where Recorded	How Often Recorded	Disposition of Data[a]
Dates and times of test events	ETV data sheets	Start/end of test and at each change of a test parameter	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
Sample collection and preparation information, including chain-of-custody	ETV data sheets and chain-of-custody forms	At time of sample collection and preparation	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
R.A.P.I.D.® System procedures and sample results	ETV data sheets and data acquisition system	Throughout test duration	Manually incorporated in data spreadsheets
Enumeration data	Enumeration data forms and ETV data sheets	With every enumeration	Used to organize/check test results
Reference method procedures and sample results	Data acquisition system, as appropriate	Throughout sample analysis process	Transferred to spreadsheets

[a] All activities subsequent to data recording were carried out by Battelle, except for the reference method analyses (DW characterization), which were carried out by ATEL.

Chapter 5
Data Analysis

The R.A.P.I.D.® System was evaluated for qualitative results (i.e., positive/negative responses to samples) based on the expected application of rapid PCR technologies as rapid screening tools. All data analyses were based on these qualitative results. QC and MB samples were not included in any of the analyses.

5.1 Accuracy

Accuracy was assessed by evaluating how often the R.A.P.I.D.® System results were positive in the presence of a concentration of contaminant above the system LOD. Contaminant-only PT samples were used for this analysis. An overall percent agreement was determined by dividing the number of positive responses by the overall number of analyses of contaminant-only PT samples above the system LOD.

5.2 Specificity

The ability of the R.A.P.I.D.® System to provide a negative response when the contaminant was absent was assessed. The specificity rate was determined by dividing the number of negative responses by the total number of unspiked samples.

5.3 False Positive/Negative Responses

A false positive response was defined as a detectable or positive R.A.P.I.D.® System response when the ASTM Type II DI water (including interferent samples) or DW samples were not spiked. A false positive rate was reported as the frequency of false positive results out of the total number of unspiked samples.

A false negative response was defined as a non-detectable response or negative response when the sample was spiked with a contaminant at a concentration greater than the system LOD. Spiked PT (contaminant and interferent) samples and spiked DW samples were included in the analysis. A false negative rate was evaluated as the frequency of false negative results out of the total number of spiked samples for a particular contaminant.

5.4 Precision

The precision of the four replicates of each sample set were assessed. Responses were considered consistent if all four replicates gave the same result. The precision of the R.A.P.I.D.® System was assessed by calculating the overall number of consistent responses for all the sample sets.

5.5 Interferences

The potential effect of the DW matrix on the R.A.P.I.D.® System performance was evaluated qualitatively by comparing the results for the spiked and unspiked DW samples to those for the PT samples. Similarly, the potential effect of interferent PT samples containing humic and fulvic acids at two levels, both spiked and not spiked with bacteria, were evaluated.

5.6 Other Performance Factors

Aspects of the R.A.P.I.D.® System performance such as ease of use and sample throughput are discussed in Section 6. Also addressed are qualitative observations of the verification staff pertaining to the performance of the R.A.P.I.D.® System.

Chapter 6
Test Results

The results for the R.A.P.I.D.® System were evaluated based on the responses provided by the R.A.P.I.D.® software Advanced Options LCDA quantification analysis Second Derivative Maximum output. The R.A.P.I.D.® software offers both an Advanced Options/LCDA as well as a simplified Detector window to view the PCR results. The Detector view is an auto-analysis feature that provides a "Present"/"Not Detected" response for the sample in each capillary in a single table. The Advanced Options interface provides more information about the amplification of each sample, including fluorescence curves and crossing points. In a testing scenario, if the Detector option was used and a "Present" response was given, Idaho Technology would train the user to proceed to the LCDA view to further evaluate the data. In the interest of maintaining consistency in data interpretation, the LCDA interface was used for the interpretation of all sample results.

An example amplification plot from the LCDA analysis for *B. anthracis* is presented in Figure 6-1. The plot displays the fluorescence versus the thermal cycler cycle number. In this example, all of the samples are displayed at once and assigned a different color. Using the R.A.P.I.D.® software, the number of samples displayed on the amplification plot can be controlled by the operator. Only qualitative (positive/negative) responses were recorded for each sample. To determine the results of each sample, the crossing point values as well as the amplification curves of each sample were monitored. The crossing point value, a fractional cycle number where the sample fluorescence is differentiated from the background fluorescence, is determined by the software and based on the second derivative maximum of the amplification curve, or the point on the curve where the rate of fluorescence changes the fastest. A sample was considered positive (bacteria detected in the sample) if a crossing point was assigned to that sample and the amplification curve for that sample was above the baseline. A sample was considered negative (no bacteria detected in the sample) when no crossing point value was assigned by the software and the amplification curve was along the baseline with no sign of exponential increase. A sample was also considered negative if a crossing point value was assigned to the sample but no amplification above the baseline was apparent for that sample's amplification curve. Samples were considered positive or negative only if both capillaries from the split analysis of the sample showed positive or negative results. For the purposes of this test, amplification curves that were above the baseline and assigned a crossing point by the software were considered positive, regardless of the cycle number at which they crossed. In some cases, many crossing point values were high (i.e., close to 40 cycles). Often, such high values,

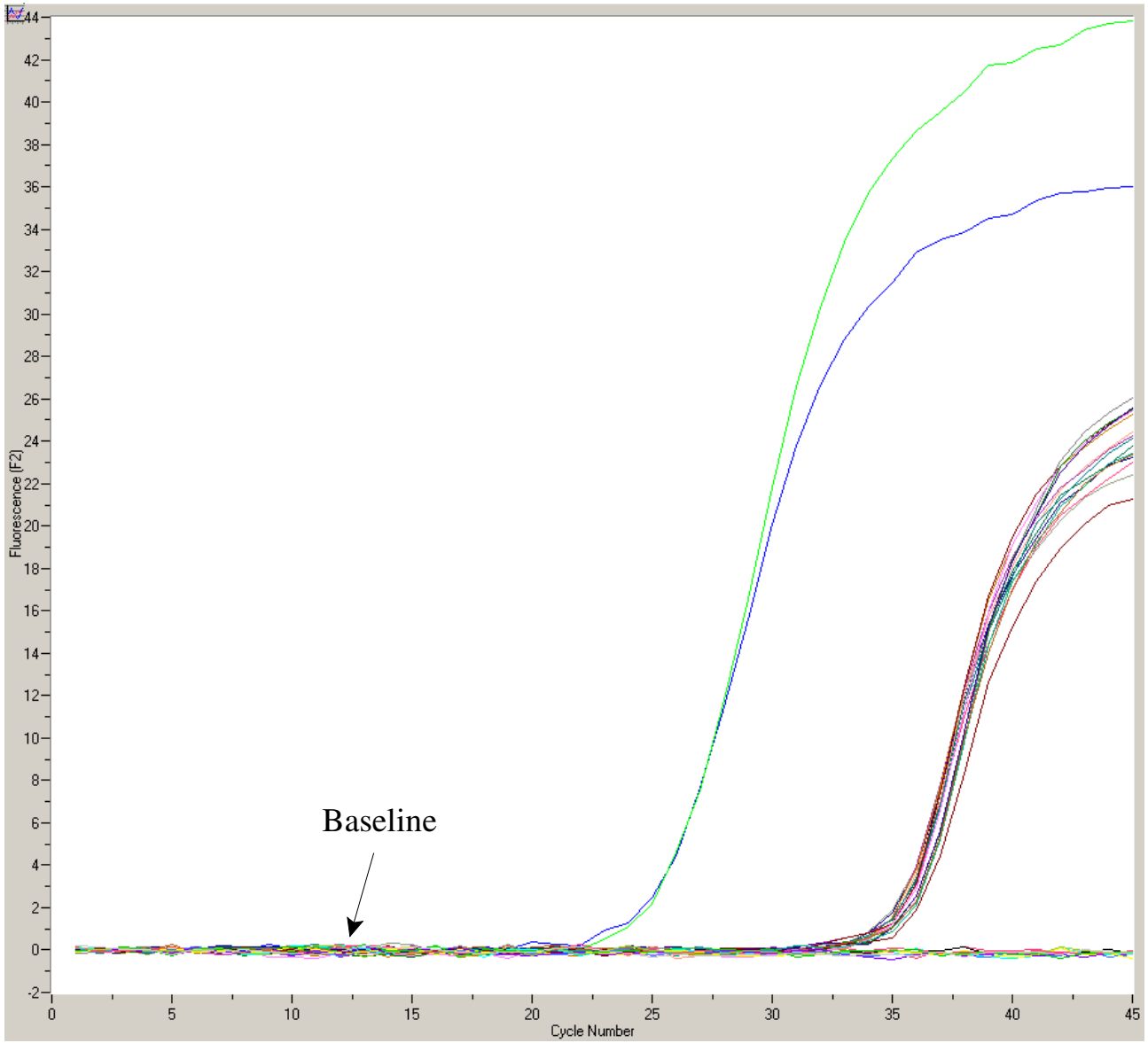

Figure 6-1. LCDA Amplification Plot Samples Analyzed Using *B. anthracis* Target 2. The amplification for each sample is represented by a different color.

particularly when the duplicate (or split) samples do not agree and amplification is only slightly above the baseline, might lead to suspect amplification and might not be considered by some to be a conclusive positive sample without further analyses. Results for a sample were deemed inconclusive when the two capillaries for the sample did not agree with each other (one was positive and one was negative). For the purposes of this test, such samples were not considered positive or negative. In a real-world scenario, Idaho Technology recommends that samples with inconclusive results be rerun to confirm the presence or absence of the bacteria of interest.

Positive and negative controls were monitored with each set of samples placed on the R.A.P.I.D.® 7200 instrument. No positive controls failed. Three negative controls were unsuccessful throughout the entire verification test (for all bacteria). When the negative controls failed, the samples were prepared again (using the freeze-dried reagents), loaded onto the carousel, and run again.

6.1 Accuracy

The results for the R.A.P.I.D.® System using the contaminant-only PT samples containing *F. tularensis*, *Y. pestis*, *B. anthracis*, *Brucella suis*, and *E. coli* are discussed in this section. The infective/lethal dose samples for each bacteria are included in the contaminant-only PT samples. In the case of *Y. pestis*, *B. anthracis*, *Brucella suis*, and *E. coli*, the infective/lethal doses (see Table 3-1) were below the vendor-stated system LOD. The results for each bacteria at the infective/lethal dose are presented in the following tables, but those for *Y. pestis*, *B. anthracis*, *Brucella suis*, and *E. coli* were not included in the overall accuracy calculations for those bacteria.

6.1.1 F. tularensis

Two targets were tested for samples containing *F. tularensis*, Target 1 and Target 2. The results obtained for the PT samples containing *F. tularensis* for both targets are given in Table 6-1a. All concentration levels analyzed for both targets generated 4 out of 4 positive responses for each set of replicate samples. An overall percent agreement was determined by dividing the number of positive responses by the overall number of analyses of contaminant-only PT samples. This resulted in 100% agreement for the overall accuracy of the R.A.P.I.D.® System for the detection of *F. tularensis* using both Target 1 and Target 2 reagents.

6.1.2 Y. pestis

The R.A.P.I.D.® System has two assays for *Y. pestis*, Target 1 and Target 2. The results obtained for the PT samples containing *Y. pestis* in both targets are given in Table 6-1b. All samples with concentration levels above the vendor-stated system LOD generated positive responses using both Target 1 and Target 2. The infective/lethal dose of *Y. pestis* (0.28 cfu/mL) was below the R.A.P.I.D.® System LOD for this bacteria and produced no positive responses in four replicates in either target. An overall percent agreement was determined by dividing the number of positive

Table 6-1a. *F. tularensis* Contaminant-Only PT Sample Results

Sample Type	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates	
		Target 1	Target 2
PT Samples	4×10^5 [b]	4/4	4/4
	2×10^3	4/4	4/4
	5×10^3	4/4	4/4
	1×10^4	4/4	4/4
	5×10^4	4/4	4/4
Overall Accuracy		**100% (20/20)**	**100% (20/20)**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] Infective/lethal dose.

Table 6-1b. *Y. pestis* Contaminant-Only PT Sample Results

Sample Type	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates	
		Target 1	Target 2
PT Samples	0.28 [b]	0/4	0/4
	2×10^3	4/4	4/4
	5×10^3	4/4	4/4
	1×10^4	4/4	4/4
	5×10^4	4/4	4/4
Overall Accuracy		**100% (16/16)[c]**	**100% (16/16)[c]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *Y. pestis*.
[c] Excludes infective/lethal dose concentration, which was below the system LOD.

responses by the overall number of analyses of contaminant-only PT samples above the R.A.P.I.D.® System LOD. This resulted in 100% agreement for the overall accuracy of the R.A.P.I.D.® System in detecting *Y. pestis* above the R.A.P.I.D.® System LOD using both Target 1 and Target 2 reagents.

6.1.3 B. anthracis

Three R.A.P.I.D.® System assays were tested for *B. anthracis*: Target 1, Target 2, and Target 3. The results obtained for the PT samples containing *B. anthracis* are given in Table 6-1c. The infective/lethal dose of *B. anthracis* was below the R.A.P.I.D.® System LOD for this bacteria. An overall percent agreement was determined by dividing the number of positive responses by the overall number of analyses of contaminant-only PT samples above the R.A.P.I.D.® System LOD. Thus, the infective/lethal dose of *B. anthracis* was not included in this calculation.

Contaminant-only PT samples at 5, 10, and 50 times the vendor-provided system LOD generated all positive responses for Target 1. Three of the four replicates at 2×10^3 cfu/mL

resulted in positive responses, while one replicate at this concentration level resulted in a positive response for one capillary and a negative response for the other capillary of the split replicate sample. This indicated inconclusive results for this replicate (i.e., the sample could be declared neither positive nor negative). Idaho Technology indicated that pipetting error when splitting the sample into two capillaries can cause mixed results for the split sample, as can Poisson distribution effects at low bacterial concentration levels. In a screening scenario, inconclusive results would lead to further testing of the sample, but this was beyond the scope of this test. The infective/lethal dose of *B. anthracis* for Target 1 reagents resulted in three inconclusive and one negative response. The overall accuracy of the R.A.P.I.D.® System using Target 1 in detecting *B. anthracis* above the R.A.P.I.D.® System LOD was 94% (15/16).

As with Target 1, PT samples at 5, 10, and 50 times the vendor-provided system LOD generated 4 out of 4 positive responses for Target 2. Three of the four replicates at 2×10^3 cfu/mL resulted in positive responses, while one replicate at this concentration level resulted in a negative response. At the infective/lethal dose for Target 2, two replicates produced inconclusive results, while the remaining two replicates resulted in negative responses. The overall accuracy of the R.A.P.I.D.® System using Target 2 in detecting *B. anthracis* above the R.A.P.I.D.® System LOD was 94% (15/16).

Table 6-1c. *B. anthracis* Contaminant-Only PT Sample Results

		Inconclusive Results[c]			**Positive Results Out of Total Replicates**		
Sample Type	**Concentration[a] (cfu/mL)**	**Target 1**	**Target 2**	**Target 3**	**Target 1**	**Target 2**	**Target 3**
PT Samples	200[b]	3	2	2	0/4	0/4	0/4
	2×10^3	1	0	0	3/4	3/4	4/4[e]
	5×10^3	0	0	0	4/4	4/4	4/4
	1×10^4	0	0	0	4/4	4/4	4/4
	5×10^4	0	0	0	4/4	4/4	4/4
Overall Accuracy[d]					**94% (15/16)**	**94% (15/16)**	**100% (16/16)**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).

[b] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *B. anthracis*.

[c] Inconclusive results are one positive and one negative result for each capillary of the split sample for a given replicate. They were considered neither positive nor negative.

[d] Excludes infective/lethal dose concentration, which was below the system LOD.

[e] Samples at 2×10^3 cfu/mL had to be rerun for Target 3 because of suspected sample preparation problems.Only the rerun results are presented here.

Using Target 3, all samples containing *B. anthracis* at concentration levels above the vendor-stated system LOD generated 4 out of 4 positive responses. The infective/lethal dose replicates for Target 3 reagents resulted in two inconclusive and two negative responses. The overall accuracy of the R.A.P.I.D.® System using Target 3 in detecting *B. anthracis* above the R.A.P.I.D.® System LOD was 100% (16/16).

6.1.4 Brucella suis

One R.A.P.I.D.® System target was tested for *Brucella suis*. The results obtained for the PT samples containing *Brucella suis* are given in Table 6-1d. Contaminant-only PT samples at 5, 10, and 50 times the vendor-provided system LOD generated all positive responses. Two of the four replicates at 2×10^3 cfu/mL resulted in positive responses, while the remaining two replicates at this concentration level resulted in inconclusive responses (one positive and one negative for each split sample). The infective/lethal dose of *Brucella suis* was below the R.A.P.I.D.® System LOD for this bacteria. The infective/lethal dose replicates generated three negative and one inconclusive response.

The overall accuracy in detecting *Brucella suis* above the R.A.P.I.D.® System LOD was 88% (14/16). The infective/lethal dose of *Brucella suis* was not included in this calculation because it was below the R.A.P.I.D.® System LOD for this bacteria.

Table 6-1d. *Brucella suis* Contaminant-Only PT Sample Results

Sample Type	Concentration[a] (cfu/mL)	Inconclusive Results[c]	Positive Results Out of Total Replicates
PT Samples	40[b]	1	0/4
	2×10^3	2	2/4
	5×10^3	0	4/4
	1×10^4	0	4/4
	5×10^4	0	4/4
Overall Accuracy			**88% (14/16)[d]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *Brucella suis*.
[c] Inconclusive results are one positive and one negative result for each capillary of the split sample for a given replicate. They were considered neither positive nor negative.
[d] Excludes infective/lethal dose concentration, which was below the system LOD.

6.1.5 E. coli

One R.A.P.I.D.® System target was tested for *E. coli*. The results obtained for the PT samples containing *E. coli* are given in Table 6-1e. All samples with concentration levels above the vendor-stated system LOD generated all positive responses. The infective/lethal dose of *E. coli*

(0.2 cfu/mL) was below the R.A.P.I.D.® System LOD for this bacteria and produced no positive responses in four replicates. All responses at the infective/lethal dose were negative. There was 100% agreement for the overall accuracy in detecting *E. coli* above the R.A.P.I.D.® System LOD.

Table 6-1e. *E. coli* Contaminant-Only PT Sample Results

Sample Type	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
PT Samples	0.2[b]	0/4
	2×10^3	4/4
	5×10^3	4/4
	1×10^4	4/4
	5×10^4	4/4
Overall Accuracy		**100% (16/16)[c]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *E. coli*.
[c] Excludes infective/lethal dose concentration, which was below the system LOD.

6.2 Specificity

Specificity assesses the R.A.P.I.D.® System's ability to provide a negative response when the contaminant was absent. The results from all unspiked interferent PT samples and unspiked DW samples are presented in this section. Negative results out of total replicates are presented in each table.

6.2.1 F. tularensis

The results obtained for *F. tularensis* Target 1 and Target 2 for the unspiked interferent and DW samples are given in Table 6-2a. All unspiked DW samples for Target 1 showed negative responses. For the interferent samples, all four replicates of unspiked 0.5 mg/L humic and fulvic acids generated negative results. Only the unspiked 2.5 mg/L humic and fulvic acid replicates for Target 1 showed inconsistent negative responses. Three of the replicates for unspiked 2.5 mg/L humic and fulvic acids had one positive and one negative response for each capillary of the split sample. This indicated inconclusive results for these replicates (i.e., the sample could be declared neither positive nor negative). In general, the positive results of the split samples (for the inconclusive results) had late-cycle crossing points (~38 cycles) with the fluorescence barely above the baseline for many of the results. The fourth replicate was negative for this set.

All unspiked interferent PT samples showed negative responses for Target 2. Similarly, OH, FL, and NY unspiked DW samples showed negative responses, indicating that the bacteria was not

present in these samples, as would be expected. For the unspiked CA DW samples, one replicate resulted in one positive and one negative response for the split sample. This indicated inconclusive results for this replicate. The remaining three replicates were negative. The CA DW was further analyzed to determine the presence (or absence) of *F. tularensis* naturally in the water (see section 3.3.2). *F. tularensis* could not be identified in the sample.

An overall specificity rate was determined by dividing the number of negative responses by the overall number of analyses of unspiked samples. This resulted in 88% agreement for the overall specificity of the R.A.P.I.D.® System for *F. tularensis* Target 1, and 96% agreement for the overall specificity for Target 2.

Table 6-2a. *F. tularensis* Specificity Results

		Negative Results Out of Total Replicates	
Sample Type	**Sample**	**Target 1**	**Target 2**
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid, unspiked	4/4	4/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid, unspiked	1/4[(a)]	4/4
DW Samples	OH DW, unspiked	4/4	4/4
	CA DW, unspiked	4/4	3/4[(b)]
	FL DW, unspiked	4/4	4/4
	NY DW, unspiked	4/4	4/4
Overall Specificity		**88% (21/24)[(a)]**	**96% (23/24)[(b)]**

[(a)] Three 2.5 mg/L humic and fulvic acid replicates had one positive and one negative result for each capillary of the split sample. These were inconclusive results.

[(b)] One CA DW replicate had one positive and one negative results for each capillary of the split sample. This indicated an inconclusive result.

6.2.2 Y. pestis

The results obtained for *Y. pestis* Target 1 and Target 2 for the analysis of unspiked interferent PT and DW samples are given in Table 6-2b. For both targets, all unspiked interferent PT samples and unspiked DW samples generated negative responses for all of the replicates. This resulted in 100% agreement for the overall specificity of the R.A.P.I.D.® System for *Y. pestis* Target 1 and Target 2.

Table 6-2b. *Y. pestis* Specificity Results

Sample Type	Sample	Negative Results Out of Total Replicates	
		Target 1	**Target 2**
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid, unspiked	4/4[(a)]	4/4[(a)]
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid, unspiked	4/4	4/4
DW Samples	OH DW, unspiked	4/4	4/4
	CA DW, unspiked	4/4	4/4
	FL DW, unspiked	4/4	4/4
	NY DW, unspiked	4/4	4/4
Overall Specificity		**100% (24/24)**	**100% (24/24)**

[(a)] These samples were rerun because of suspected sample preparation problems. Only the rerun results are presented here.

6.2.3 B. anthracis

The results obtained using *B. anthracis* Targets 1, 2, and 3 for the analysis of unspiked interferent and DW samples are given in Table 6-2c. All unspiked interferent PT samples and unspiked DW samples showed negative responses for all of the replicates for all three targets. An overall specificity rate was determined by dividing the number of negative responses by the overall number of analyses of unspiked samples. This resulted in 100% agreement for the overall specificity of the R.A.P.I.D.® System for each target (Targets 1, 2, and 3) for *B. anthracis*.

6.2.4 Brucella suis

The results obtained for the *Brucella suis* assay for the analysis of unspiked interferent and DW samples are given in Table 6-2d. All unspiked interferent PT samples and unspiked DW samples showed negative responses for all of the replicates. This resulted in 100% agreement for the overall specificity of the R.A.P.I.D.® System for *Brucella suis*.

Table 6-2c. *B. anthracis* Specificity Results

Sample Type	Sample	Negative Results Out of Total Replicates		
		Target 1	**Target 2**	**Target 3**
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid, unspiked	4/4	4/4	4/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid, unspiked	4/4	4/4	4/4
DW Samples	OH DW, unspiked	4/4	4/4	4/4
	CA DW, unspiked	4/4	4/4	4/4
	FL DW, unspiked	4/4	4/4	4/4
	NY DW, unspiked	4/4	4/4	4/4
Overall Specificity		**100% (24/24)**	**100% (24/24)**	**100% (24/24)**

Table 6-2d. *Brucella suis* Specificity Results

Sample Type	Sample	Negative Results Out of Total Replicates
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid, unspiked	4/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid, unspiked	4/4
DW Samples	OH DW, unspiked	4/4
	CA DW, unspiked	4/4
	FL DW, unspiked	4/4
	NY DW, unspiked	4/4
Overall Specificity		**100% (24/24)**

6.2.5 E. coli

The results obtained for the *E. coli* assay for unspiked interferent and DW samples are given in Table 6-2e. All unspiked interferent PT samples and unspiked DW samples showed negative responses for all of the replicates. An overall specificity rate was determined by dividing the

number of negative responses by the overall number of analyses of unspiked samples. This resulted in 100% agreement for the overall specificity of the R.A.P.I.D.® System for *E. coli*.

Table 6-2e. *E. coli* Specificity Results

Sample Type	Sample	Negative Results Out of Total Replicates
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid, unspiked	4/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid, unspiked	4/4
DW Samples	OH DW, unspiked	4/4
	CA DW, unspiked	4/4
	FL DW, unspiked	4/4
	NY DW, unspiked	4/4
Overall Specificity		**100% (24/24)**

6.3 False Positive/Negative Responses

Contaminant-only PT samples, interferent PT samples, and DW samples were evaluated to determine false positive and false negative results for the R.A.P.I.D.® System. Included in the calculations were the 16 additional interferent sample replicates (0.5 mg/L humic and fulvic acids) per bacteria tested to determine the effects of the DNA extraction and purification on the results. A false positive response was defined as a positive result when bacteria were not spiked into the sample. A false negative response was defined as a negative result when the sample was spiked with a contaminant at a concentration greater than the R.A.P.I.D.® System LOD for that bacteria.

It should be noted that false positive responses cannot be absolutely confirmed as false because there is a possibility of cross-contamination. All appropriate steps were taken throughout the verification test to avoid this issue by using three separate work areas, by following daily cleanup procedures, and by loading controls and samples into the capillaries in the appropriate sequence (negative controls, samples, and then positive controls). However, cross-contamination is always a possibility in any PCR process.[12] No appropriate reference method was available to cross-check the amplified PCR product to confirm the R.A.P.I.D.® System responses. When sample preparation error was suspected (e.g., sample appeared to be unspiked when it should have been spiked, or spiked when it should have been blank), the sample was re-evaluated. If sample preparation errors or cross-contamination were suspected after re-analysis, only the results of the reruns were presented.

6.3.1 F. tularensis

Tables 6-3a and 6-3b present *F. tularensis* Target 1 and Target 2 (respectively) false negative and false positive results. The number of positive samples out of the total replicates analyzed is presented in each table. Total negative and inconclusive results are also presented for each set of replicates. No false positive samples were found in any of the sample matrices for either target. No false negative results were found for Target 1. Four false negatives were found for Target 2: one in a set of replicates for spiked 0.5 mg/L humic and fulvic acids, and three in a set of replicates for spiked CA DW. Three replicates for unspiked 2.5 mg/L humic and fulvic acids showed one negative and one positive result for each of the split sample for Target 1. Because both results for a split sample did not agree, the results for the replicates were determined to be inconclusive. Inconclusive results were also found for one replicate in spiked CA DW and one replicate in unspiked CA DW using Target 2.

6.3.2 Y. pestis

Table 6-3c presents the false negative and false positive results for *Y. pestis* Target 1 and Target 2. The number of positive samples out of the total replicates analyzed is presented in the table. No false positive or false negative samples were found in any of the sample matrices for either target. No inconclusive results were found in any of the sample matrices for either target.

6.3.3 B. anthracis

Tables 6-3d, 6-3e, and 6-3f present the false positive/negative results for *B. anthracis* Targets 1, 2, and 3 (respectively). The number of positive samples out of the total replicates analyzed is presented in each table. The number of negative and inconclusive responses for each set of replicates is also presented. No false positives were found in any of the sample matrices for Targets 1, 2, and 3. For Target 1, two false negatives were found: one negative response for a spiked NY DW replicate and one negative response for a spiked CA DW replicate. One replicate each for spiked FL DW, spiked CA DW, and spiked DI water at 2×10^3 cfu/mL showed one positive and one negative response for the split sample, indicating a neither fully positive nor fully negative response. The results for these replicates were determined to be inconclusive.

Target 2 also had two false negatives: one replicate of 2×10^3 cfu/mL *B. anthracis* in DI water and one replicate of spiked CA DW. Two inconclusive results were also found for spiked CA DW replicates using Target 2 reagents. Target 3 reported no false negative results, although all four spiked CA DW replicates generated inconclusive results.

6.3.4 Brucella suis

Table 6-3g presents the false negative/false positive results for *Brucella suis*. The number of positive samples out of the total replicates analyzed is presented in the table. No false positive or false negative samples were found in any of the sample matrices. Two replicates for DI water spiked at 2×10^3 cfu/mL showed one negative and one positive result for each of the split samples. Because both results for a split sample did not agree, the results for these two replicates were determined to be inconclusive.

Table 6-3a. *F. tularensis* Target 1 False Positive/Negative Results

Sample Type	Sample	Conc. [a] (cfu/mL)	Negative Results	Inconclusive Results [b]	Positive Results Out of Total Replicates
Contaminant-Only PT Samples	DI water	4×10^{5} [c]	0	0	4/4
	DI water	2×10^{3} [d]	0	0	4/4
	DI water	5×10^{3}	0	0	4/4
	DI water	1×10^{4}	0	0	4/4
	DI water	5×10^{4}	0	0	4/4
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	4	0	0/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^{4}	0	0	20/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	1	3	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^{4}	0	0	4/4
DW Samples	OH DW	Blank	4	0	0/4
	OH DW	1×10^{4}	0	0	4/4
	CA DW	Blank	4	0	0/4
	CA DW	1×10^{4}	0	0	4/4[d]
	FL DW	Blank	4	0	0/4
	FL DW	1×10^{4}	0	0	4/4
	NY DW	Blank	4	0	0/4
	NY DW	1×10^{4}	0	0	4/4
False Positive Rate					**0/24**
False Negative Rate					**0/60**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).

[b] Inconclusive results are one positive and one negative result for each capillary of the split sample for a given replicate. They were considered neither positive nor negative.

[c] Infective/lethal dose.

[d] Spiked CA DW samples were rerun because of suspected cross-contamination problems. Only the rerun results are presented here.

Table 6-3b. *F. tularensis* Target 2 False Positive/Negative Results

Sample Type	Sample	Conc.[a] (cfu/mL)	Negative Results[b]	Inconclusive Results[c]	Positive Results Out of Total Replicates
Contaminant-Only PT Samples	DI water	4×10^{5}[d]	0	0	4/4
	DI water	2×10^{3}	0	0	4/4
	DI water	5×10^{3}	0	0	4/4
	DI water	1×10^{4}	0	0	4/4
	DI water	5×10^{4}	0	0	4/4
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	4	0	0/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^{4}	1	0	19/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	4	0	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^{4}	0	0	4/4
DW Samples	OH DW	Blank	4	0	0/4
	OH DW	1×10^{4}	0	0	4/4
	CA DW	Blank	3	1	0/4
	CA DW	1×10^{4}	3	1	0/4[e]
	FL DW	Blank	4	0	0/4
	FL DW	1×10^{4}	0	0	4/4
	NY DW	Blank	4	0	0/4
	NY DW	1×10^{4}	0	0	4/4
False Positive Rate					**0/24**
False Negative Rate					**4/60**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] False negatives are shaded.
[c] Inconclusive results are one positive and one negative result for each capillary of the split sample for a given replicate. They were considered neither positive nor negative.
[d] Infective/lethal dose.
[e] Spiked CA DW samples were rerun because of suspected cross-contamination problems. Only the rerun results are presented here.

Table 6-3c. *Y. pestis* False Positive/Negative Results

Sample Type	Sample	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates	
			Target 1	Target 2
Contaminant-Only PT Samples	DI water	2×10^3	4/4	4/4
	DI water	5×10^3	4/4	4/4
	DI water	1×10^4	4/4	4/4
	DI water	5×10^4	4/4	4/4
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	0/4[b]	0/4[b]
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^4	20/20[c]	20/20[c]
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	0/4	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^4	4/4	4/4
DW Samples	OH DW	Blank	0/4	0/4
	OH DW	1×10^4	4/4	4/4
	CA DW	Blank	0/4	0/4
	CA DW	1×10^4	4/4	4/4
	FL DW	Blank	0/4	0/4
	FL DW	1×10^4	4/4	4/4
	NY DW	Blank	0/4	0/4
	NY DW	1×10^4	4/4	4/4
False Positive Rate			**0/24**	**0/24**
False Negative Rate			**0/56[c]**	**0/56[d]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).

[b] Spiked 0.5 mg/L humic and fulvic acid samples were rerun because of suspected cross-contamination problems. Only the rerun results are presented here.

[c] One set of replicate 0.5 mg/L humic and fulvic acid samples had to be rerun because of suspected cross-contamination problems. Only the rerun results are presented here.

[d] The infective/lethal dose for *Y. pestis* was below the system LOD and thus not included in this calculation.

Table 6-3d. *B. anthracis* Target 1 False Positive/Negative Results

Sample Type	Sample		Conc.[a] (cfu/mL)	Negative Results[b]	Inconclusive Results[c]	Positive Results Out of Total Replicates
Contaminant-Only PT Samples	DI water		2×10^3	0	1	3/4
	DI water		5×10^3	0	0	4/4
	DI water		1×10^4	0	0	4/4
	DI water		5×10^4	0	0	4/4
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid		Blank	4	0	0/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid		1×10^4	0	0	20/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid		Blank	4	0	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid		1×10^4	0	0	4/4
DW Samples	OH DW		Blank	4	0	0/4
	OH DW	0	1×10^4		0	4/4
	CA DW		Blank	4	0	0/4
	CA DW	1	1×10^4		1	2/4[d]
	FL DW		Blank	4	0	0/4
	FL DW	0	1×10^4		1	3/4
	NY DW		Blank	4	0	0/4
	NY DW	1	1×10^4		0	3/4
False Positive Rate						**0/24**
False Negative Rate						**2/56[e]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] False negatives are shaded.
[c] Inconclusive results are one positive and one negative result for each capillary of the split sample for a given replicate. They were considered neither positive nor negative.
[d] These samples were rerun because of suspected cross-contamination problems. Only the rerun results are presented here.
[e] The infective/lethal dose for *B. anthracis* was below the system LOD and thus not included in this calculation.

Table 6-3e. *B. anthracis* Target 2 False Positive/Negative Results

Sample Type	Sample	Conc.[a] (cfu/mL)	Negative Results[b]	Inconclusive Results[c]	Positive Results Out of Total Replicates
Contaminant-Only PT Samples	DI water	2×10^{3}[d]	1	0	3/4
	DI water	5×10^{3}	0	0	4/4
	DI water 0	1×10^{4}		0	4/4
	DI water 0	5×10^{4}		0	4/4
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	4	0	0/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^{4}	0	0	20/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	4	0	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^{4}	0	0	4/4
DW Samples	OH DW	Blank	4	0	0/4
	OH DW	1×10^{4}	0	0	4/4
	CA DW	Blank	4	0	0/4
	CA DW 1	1×10^{4}		2	1/4[d]
	FL DW	Blank	4	0	0/4
	FL DW	1×10^{4}	0	0	4/4
	NY DW	Blank	4	0	0/4
	NY DW	1×10^{4}	0	0	4/4
False Positive Rate					**0/24**
False Negative Rate					**2/56[e]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] False negatives are shaded.
[c] Inconclusive results are one positive and one negative result for each capillary of the split sample for a given replicate. They were considered neither positive nor negative
[d] These samples were rerun because of suspected cross-contamination problems. Only the rerun results are presented here.
[e] The infective/lethal dose for *B. anthracis* was below the system LOD and thus not included in this calculation.

Table 6-3f. *B. anthracis* Target 3 False Positive/Negative Results

Sample Type	Sample	Conc.[a] (cfu/mL)	Negative Results	Inconclusive Results[b]	Positive Results Out of Total Replicates
Contaminant-Only PT Samples	DI water	2×10^{3}[c]	0	0	4/4
	DI water	5×10^{3}	0	0	4/4
	DI water	1×10^{4}	0	0	4/4
	DI water	5×10^{4}	0	0	4/4
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	4	0	0/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^{4}	0	0	20/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	4	0	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^{4}	0	0	4/4
DW Samples	OH DW	Blank	4	0	0/4
	OH DW0	1×10^{4}		0	4/4
	CA DW	Blank	4	0	0/4
	CA DW	0 1×10^{4}		4	0/4[c]
	FL DW	Blank	4	0	0/4
	FL DW 0	1×10^{4}		0	4/4
	NY DW	Blank	4	0	0/4
	NY DW0	1×10^{4}		0	4/4
False Positive Rate					**0/24**
False Negative Rate					**0/56[d]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).

[b] Inconclusive results are one positive and one negative result for each capillary of the split sample for a given replicate. They were considered neither positive nor negative.

[c] These samples were rerun because of suspected cross-contamination problems. Only the rerun results are presented here.

[d] The infective/lethal dose for *B. anthracis* was below the system LOD and thus not included in this calculation.

Table 6-3g. *Brucella suis* False Positive/Negative Results

Sample Type	Sample	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
Contaminant-Only PT Samples	DI water	2×10^3	2/4[b]
	DI water	5×10^3	4/4
	DI water	1×10^4	4/4
	DI water	5×10^4	4/4
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	0/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^4	20/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^4	4/4
DW Samples	OH DW	Blank	0/4
	OH DW	1×10^4	4/4
	CA DW	Blank	0/4
	CA DW	1×10^4	4/4
	FL DW	Blank	0/4
	FL DW	1×10^4	4/4
	NY DW	Blank	0/4
	NY DW	1×10^4	4/4
False Positive Rate			**0/24**
False Negative Rate			**0/56[b,c]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).

[b] Two 2×10^3 cfu/mL replicates had one positive and one negative result for each capillary of the split sample. This indicated inconclusive results.

[c] The infective/lethal dose for *Brucella suis* was below the system LOD and thus not included in this calculation.

6.3.5 E. coli

Table 6-3h presents the false negative and false positive results for *E. coli*. The number of positive samples out of the total replicates analyzed is presented in the table. No false positive or false negative samples were found in any of the sample matrices.

Table 6-3h. *E. coli* False Positive/Negative Results

Sample Type	Sample	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
Contaminant-Only PT Samples	DI water	2×10^3	4/4
	DI water	5×10^3	4/4
	DI water	1×10^4	4/4
	DI water	5×10^4	4/4
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	0/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^4	16/16 [b]
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^4	4/4
DW Samples	OH DW	Blank	0/4
	OH DW	1×10^4	4/4
	CA DW	Blank	0/4
	CA DW	1×10^4	4/4
	FL DW	Blank	0/4
	FL DW	1×10^4	4/4
	NY DW	Blank	0/4
	NY DW	1×10^4	4/4
False Positive Rate			**0/24**
False Negative Rate			**0/52[b, c]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] One set of spiked 0.5 mg/L humic and fulvic acid replicates were suspected of having cross-contamination problems. The samples were not rerun, so the results are not reported here for consistency with other rerun reporting.
[c] The infective/lethal dose for *E. coli* was below the system LOD and thus not included in this calculation.

6.4 Precision

The performance of the *F. tularensis* Target 1 assay within sample sets of four replicates was consistent. Only one set of replicates, that for interferent samples spiked with 2.5 mg/L humic and fulvic acids, was inconsistent, with inconclusive results for three replicates and a negative result for one replicate. All other samples showed the same results within a set of replicates. Thus, for *F. tularensis* Target 1, one of the 21 sets of replicates that were analyzed was determined to be inconsistent, indicating that 95% of the sample sets showed consistent results among the replicates.

The precision of the *F. tularensis* Target 2 for the R.A.P.I.D.® System was not as consistent as that for Target 1. Results were inconsistent within three sets of replicate samples for Target 2. In the unspiked CA DW sample, the results for one replicate were inconclusive while the other three were negative. The same was true for spiked CA DW. In one set of spiked 0.5 mg/L each humic and fulvic acids, one negative result was found amongst three positive responses. All other samples showed the same results within a set of four replicates. Thus, for *F. tularensis* Target 2, three of the 21 sets of replicates that were analyzed were determined to be inconsistent, indicating that 86% of the sample sets showed consistent results among the replicates.

The performance of the R.A.P.I.D.® System within sample sets of four replicates for *Y. pestis* Targets 1 and 2 was very consistent. For all 21 sets of replicate samples for both targets, all replicates showed the same results within the sample set. Thus, for *Y. pestis* Targets 1 and 2, 100% of the sample sets showed consistent results among the replicates.

As with *F. tularensis*, the consistency of the sample sets for *B. anthracis* were mixed for the three targets that were evaluated. For *B. anthracis* Target 1, five sample sets had inconsistent results. These samples included two PT samples (2 x 10^3 cfu/mL and the infective/lethal dose), the spiked CA DW, the spiked FL DW, and the spiked NY DW samples. For the infective/lethal dose of *B. anthracis*, three of the four samples had inconclusive results, while one sample was negative. The infective dose of *B. anthracis* was below the LOD for the R.A.P.I.D.® System for this bacteria, so the discrepancy between replicate samples likely has more to do with this fact than the actual precision of the system. At 2 x 10^3 cfu/mL, three positive results and one inconclusive result were observed. The responses for spiked CA DW were mixed, with two positive replicates, one negative, and one inconclusive replicate response. Spiked FL DW had three positive results and one inconclusive result, while spiked NY DW had three positive and one negative response for *B. anthracis*. These five inconsistent replicate sets indicate that 76% (16/21) of the sample sets analyzed for *B. anthracis* Target 1 showed consistent results among the replicates.

The number of consistent replicate sample sets for *B. anthracis* Target 2 was higher than that for Target 1. Three of the 21 replicate sets of samples showed inconsistent results for Target 2. As with Target 1, the infective/lethal dose of *B. anthracis* showed varied results, with two inconclusive and two negative responses. Again, the infective dose of *B. anthracis* was below the LOD for the R.A.P.I.D.® System for this bacteria, so the discrepancy between replicate samples likely has more to do with this fact than the actual precision of the system. Replicates at 2 x 10^3 cfu/mL again showed inconsistent results, with three positive and one negative response. The remaining inconsistent sample set was spiked CA DW. For these replicates, one was positive, one was

negative, and the remaining two were inconclusive. Overall, 86% (18/21) of the sample sets analyzed for *B. anthracis* Target 2 showed consistent results among the replicates.

The consistency within sample sets for the *B. anthracis* assays was the highest for Target 3. As with Targets 1 and 2, the results for the infective/lethal dose of anthrax were inconsistent, with two negative and two inconclusive results. The remaining 20 of the 21 sets of replicate samples showed consistent results among the replicates. All four spiked CA DW samples were inconclusive. Thus, for *B. anthracis* Target 3, one of the 21 sets of replicates that were analyzed was determined to be inconsistent, indicating that 95% (20/21) of the sample sets showed consistent results among the replicates.

For *Brucella suis*, two out of 21 sample sets were inconsistent. The replicates at 2 x 10^3 cfu/mL showed two positive and two inconclusive responses. Three of the replicates at the infective/ lethal dose were negative, while one was inconclusive, resulting in an inconsistent set of replicates. As with *B. anthracis*, the infective/lethal dose of *Brucella suis* is below the vendor-provided system LOD for this bacteria. All other sample sets showed consistent results within a set of replicates. Thus, 90% (19/21) of the sample sets showed consistent results among the replicates.

Only 20 sets of replicate samples were considered for the precision calculations for *E. coli* (see Table 6-3h). For all 20 sets of replicate samples, all replicates showed the same results within the sample set. Thus, for *E. coli*, 100% of the sample sets showed consistent results among the replicates.

6.5 Interferences

6.5.1 Interferent PT Samples

In both the 0.5 mg/L and 2.5 mg/L humic and fulvic acid solutions, both spiked with the bacteria of interest and unspiked, the R.A.P.I.D.® System provided expected results for *Y. pestis* Targets 1 and 2; *B. anthracis* Targets 1, 2, and 3; *Brucella suis*; and *E. coli*. In the absence of the bacteria, all samples for these bacteria (and all associated targets) tested negative; in the presence of the bacteria, all samples tested positive. In the case of *F. tularensis* Target 1, all unspiked 0.5 mg/L humic and fulvic acid samples returned negative results, while all spiked 0.5 and 2.5 mg/L humic and fulvic acid samples returned positive results. The unspiked 2.5 mg/L humic and fulvic acid replicates returned one negative response and three inconclusive results, where the split samples of each of these three replicates had a positive and a negative response. All positive samples had low amplification and late crossing points. The same sample DNA, when analyzed on the R.A.P.I.D.® 7200 instrument using *F. tularensis* Target 2, returned all negative results. The discrepancy between the targets could be related to the different sensitivities of the two targets and/or potential cross-contamination. The initial spiked and unspiked 0.5 mg/L and 2.5 mg/L humic and fulvic acid samples tested using *F. tularensis* Target 2 returned all positive results when the sample was spiked and all negative results when the sample was unspiked. Discrepancies in the results for the additional 0.5 mg/L spiked samples that were tested are discussed below.

As discussed in Section 3.2.1, four solutions of humic and fulvic acids at 0.5 mg/L each spiked with each contaminant at 1×10^4 cfu/mL were prepared in addition to the initial 0.5 mg/L and 2.5 mg/L humic and fulvic acid solutions. Each solution was put through the DNA extraction and purification procedure, and then four replicates from each of the four purified DNA solutions were analyzed using the R.A.P.I.D.® System. These samples were included in the verification test in an effort to evaluate the efficacy of the DNA extraction and purification procedure in the presence of inhibitory substances. These samples also contribute to the precision evaluations of the R.A.P.I.D.® System. For *F. tularensis* Target 1; *Y. pestis* Targets 1 and 2; *B. anthracis* Targets 1, 2, and 3; *Brucella suis*; and *E. coli*, all of the samples tested resulted in positive responses. Thus, 20 out of the 20 (16/16 for *E. coli*) spiked 0.5 mg/L humic and fulvic acid samples tested resulted in positive responses for each bacteria. For *F. tularensis* Target 2, one of the 20 replicates tested produced a negative instead of positive reponse.

6.5.2 Drinking Water Samples

The R.A.P.I.D.® System DW sample results for *F. tularensis*, *Y. pestis*, *B. anthracis, Brucella suis,* and *E. coli* are presented in Tables 6-3a through 6-3h. In general, the R.A.P.I.D.® System showed positive results for each set of replicates for the spiked DW samples and negative results for each set of replicates for the unspiked samples, with a few exceptions. *B. anthracis* Targets 1, 2, and 3 as well as *F. tularensis* Targets 1 and 2 generated mixed results in spiked CA DW. *B. anthracis* Target 1 also showed inconclusive and negative results in spiked FL and NY DW, respectively. For the detection of *F. tularensis* Target 2 in unspiked CA DW, one of the four replicates had an inconclusive result. Analysis of the CA DW did not indicate the presence of *F. tularensis*. The possibility of cross-contamination causing the inconclusive results for unspiked CA DW sample cannot be ruled out.

The contaminant-only PT samples at 1×10^4 cfu/mL, the level at which the DW samples were spiked, showed consistent positive responses across all bacteria. The interferent PT samples at both 0.5 mg/L and 2.5 mg/L humic and fulvic acids also spiked at 1×10^4 cfu/mL showed consistent positive responses for all replicates across all bacteria, except for four replicates total for *F. tularensis* Targets 1 and 2. The consistency of responses in these PT samples, as well as other contaminant-only PT samples above the system LOD, would seem to indicate that some of the DW matrices used in this test, in particular CA DW, may have inhibitory or other confounding effects on the PCR process for the R.A.P.I.D.® System.

6.6 Other Performance Factors

The R.A.P.I.D.® System was operated by the same Battelle technician throughout the verification test. This technician had prior PCR experience and was trained by Idaho Technology over the course of one day in the operation of the R.A.P.I.D.® System before testing began. This training included the use of the ITI 1-2-3 Flow Kit, the R.A.P.I.D.® 7200 instrument, and the freeze-dried reagents. The Battelle technician was familiar with general DNA extraction and purification techniques, PCR reagent preparation techniques, and general thermal cycler operation, as well as general PCR theory, prior to training. The overall operation of the R.A.P.I.D.® System was straightforward, and the experienced technician found the system easy to use and had no major

difficulties operating the R.A.P.I.D.® System. The R.A.P.I.D.® System is designed to be used by operators without prior experience who have only gone through training conducted by Idaho Technology. Some features of the R.A.P.I.D.® System software are designed to complement such an inexperienced user, though the Advanced Options were used in this test.

The freeze-dried reagents provided for easy PCR setup because all of the components needed to fill two capillaries were contained within one vial. The "unknown" reagents and positive and negative control reagent vials were packaged together and color coded. It was important to throughly mix the reconstituted reagents before pipetting them into the capillaries. Prior experience using pipettes would likely make the PCR setup easier and reduce potential pipetting errors. The DNA extraction procedure using the ITI 1-2-3 Flow Kit was straightforward and easy to follow, though there were a fair number steps involved in the process. The transfer pipettes provided with the ITI 1-2-3 Flow Kit were more rudimentary than typical laboratory pipettes, but they worked well because exact amounts of buffer were not needed for the extraction procedure. The glass capillaries used to hold the sample were easy to work with but posed some problems. At one point, it became difficult to put the capillaries into the slots (rotors) in the carousel, and the technician broke multiple capillaries trying to load the instrument. Capillaries also broke during two PCR runs. After instruction from Idaho Technology, the rotor tool (provided with the system) was used to clean out the capillary slots in the carousel. After the cleaning, the capillaries posed no further problems. The R.A.P.I.D.® System software was user friendly and easy to use with clearly labeled buttons. The technician easily learned to use the Advanced Options feature, which was used to evaluate the results instead of the user interface intended for inexperienced operators. In Advanced Options, the software returned crossing points for all samples that had crossed the background fluorescence, but it was up to the technician to verify that the sample was positive. An understanding of PCR theory and amplification would make such interpretations easier.

All testing was performed in a laboratory setting because the R.A.P.I.D.® System as used in this verification test is not field portable. The R.A.P.I.D.® System is intended to be field portable, and in fact the instrument itself as used for this test could be easily transported to the field. However, to perform the DNA extraction and purification of 5 mL of water, a 50 mL capacity centrifuge had to be used. For this reason, the R.A.P.I.D.® System as used in this test was not considered field portable. Three different testing areas were required in each laboratory to operate the R.A.P.I.D.® System: one for the DNA purification, one for the reagent preparation, and one for the instrument operation. All of the reagents, including those for the ITI 1-2-3 Flow Kit and the freeze-dried reagents, were stable at room temperature. The reagents came packaged in individual, vacuum-sealed foil pouches containing 20 vials (four positive controls, two negative controls, and 14 unknown vials) along with sterile PCR grade water. The R.A.P.I.D.® 7200 instrument was approximately 20 inches x 14 inches x 10 inches its own hardened case, with a backpack for carrying. It came with its own laptop, which can be placed in the backpack.

F. tularensis and *E. coli* samples were tested in a BSL-2 laboratory; while *Y. pestis*, *B. anthracis*, and *Brucella suis* were tested in a BSL-3 laboratory. Because live bacteria were being handled, special safety requirements and protocols had to be implemented in both the BSL-2 and BSL-3 laboratories. Some of these requirements impacted the analysis time for the R.A.P.I.D.® System and are inherently present in any throughput estimations for this verification test. Thus, such

performance factors mentioned here also incorporate the safety and facility requirements necessary for this test.

A total of 92 or more samples (including method blanks) were tested for each bacteria using the R.A.P.I.D.® System. On average, the DNA extraction and isolation step for between three and nine solutions took approximately 2 hours. The PCR setup steps, including reconstituting the freeze-dried reagents and loading the capillaries, took approximately 30 minutes for each batch of samples. The thermal cycler run on the R.A.P.I.D.® 7200 instrument took approximately 30 minutes for each batch of samples. The verification staff analyzed on average three to four batches of samples on the R.A.P.I.D.® 7200 instrument per day, in some instances analyzing up to six batches a day. This equated to approximately 36 to 48 duplicate samples being analyzed per day (72 to 96 actual capillaries loaded) for three to four batches of samples and 72 duplicate samples a day (144 actual capillaries loaded) for six batches, including two controls (one split positive and one split negative) for every batch of samples analyzed.

For the purposes of this test, the R.A.P.I.D.® output was monitored using the LCDA quantification view to determine the results for each sample. The Second Derivative Maximum function was used to determine the crossing points. Within the LCDA quantification view of the data, the operator has other options with which he or she can manipulate and inspect the data. A Fit Points analysis can be used to determine the crossing points for each positive sample. This option allows for more user-defined criteria for determining the crossing points. For the Fit Points analysis, the user picks the appropriate baseline adjustment method (as with the Second Derivative Maximum analysis) and then sets the noise band threshold. The software then generates the crossing points for each positive sample based on the user-defined criteria. The R.A.P.I.D.® System also offers a melt cycle and melting curve analysis to aid in product identification.

Another more simplified screen that was not used in this verification test is also available from the R.A.P.I.D.® software for interpreting the results. The software also offers a Detector view of the results at the completion of a PCR run. In this view, all of the samples from a single run on the R.A.P.I.D.® 7200 instrument are shown in one table, including the positive and negative controls. In this table, the software generates a “Present” response beside a sample when the bacteria of interest have been detected, and a “Not Detected” beside the sample when the bacteria have not been detected in the sample. The software also monitors the control samples and will indicate to the user if a batch of samples needs to be rerun because the controls failed. An example of the Detector output is shown below in Figure 6-2. An option also exists within the R.A.P.I.D.® 7200 instrument to test for multiple assays on one carousel batch. Though this option was not fully verified in this test, for one batch of samples, in an effort to conserve time, two different assays for the same bacteria (*Y. pestis)* were analyzed in the same R.A.P.I.D.® 7200 instrument run. No problems were encountered, and the setup was easy.

Brucella - Organism Present						
#	Sample Name	Control	Score	Result	SSN	Enc. ID
1	Positive Control	Positive	234	Present		
2	Positive Control	Positive	195	Present		
3	Negative Control	Negative	-8	Not Detected		
4	Negative Control	Negative	-8	Not Detected		
5	Sample 7 a1	Unknown	183	Present		
6	Sample 7 a2	Unknown	249	Present		
7	Sample 7 b1	Unknown	127	Present		
8	Sample 7 b2	Unknown	123	Present		
9	Sample 7 c1	Unknown	159	Present		
10	Sample 7 c2	Unknown	132	Present		
11	Sample 7 d1	Unknown	176	Present		
12	Sample 7 d2	Unknown	212	Present		
13	Sample 8 a1	Unknown	0	Not Detected		
14	Sample 8 a2	Unknown	-8	Not Detected		
15	Sample 8 b1	Unknown	-3	Not Detected		
16	Sample 8 b2	Unknown	-12	Not Detected		
17	Sample 8 c1	Unknown	-3	Not Detected		
18	Sample 8 c2	Unknown	-8	Not Detected		
19	Sample 8 d1	Unknown	-5	Not Detected		
20	Sample 8 d2	Unknown	-7	Not Detected		
21	Sample 9 a1	Unknown	-13	Not Detected		
22	Sample 9 a2	Unknown	-4	Not Detected		
23	Sample 9 b1	Unknown	-6	Not Detected		
24	Sample 9 b2	Unknown	-6	Not Detected		
25	Sample 9 c1	Unknown	-4	Not Detected		
26	Sample 9 c2	Unknown	-5	Not Detected		
27	Sample 9 d1	Unknown	-5	Not Detected		
28	Sample 9 d2	Unknown	-10	Not Detected		

Figure 6-2. R.A.P.I.D.® Detector View Output for Spiked (Sample 7) and Unspiked (Samples 8 and 9) *Brucella suis* Samples.

Chapter 7
Performance Summary

The R.A.P.I.D.® System results for this verification test for samples containing *F. tularensis, Y. pestis*, *B. anthracis*, *Brucella suis,* and *E. coli* are presented in Tables 7-1 through 7-9. The results for each bacteria assay are presented in a separate table. Qualitative responses for each set of sample replicates as well as accuracy, specificity, false negatives and positives, and precision are presented in each table. A summary of the other performance factors associated with the R.A.P.I.D.® System is presented at the end of this chapter. These performance factors apply to the entire system, across all bacteria.

Table 7-1. *F. tularensis* Target 1 Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	4×10^5 cfu/mL[(a)]	4/4
			2×10^3 cfu/mL	4/4
			5×10^3 cfu/mL	4/4
			1×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
	Interferent PT samples	Humic and fulvic acids	1×10^4 cfu/mL	24/24
	DW samples	Concentrated DW	1×10^4 cfu/mL	16/16
Accuracy		100% (20 out of 20) of the contaminant-only PT samples above the system LOD were positive.		
Specificity		88% (21 out of 24) of the unspiked interferent and DW samples were negative. Three unspiked 2.5 mg/L each humic and fulvic acid replicates returned inconclusive results.[(b)]		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples. Three unspiked 2.5 mg/L each humic and fulvic acid replicates returned inconclusive results.[(b)]		
False negatives		No false negative results were obtained from the analysis of samples spiked with levels of *F. tularensis* above the system LOD.		
Precision		95% (20 out of 21) of the sample sets showed consistent results among the individual replicates within that set.[(b)]		

[(a)] Infective/lethal dose.

[(b)] Three unspiked 2.5 mg/L each humic and fulvic acid replicates had one positive and one negative result in the split samples. These were inconclusive results and would require re-analysis in a real-world scenario. The remaining replicate was negative.

Table 7-2. *F. tularensis* Target 2 Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	4×10^5 cfu/mL[(a)]	4/4
			2×10^3 cfu/mL	4/4
			5×10^3 cfu/mL	4/4
			1×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
	Interferent PT samples	Humic and fulvic acids	1×10^4 cfu/mL	23/24[(b)]
	DW samples	Concentrated DW	1×10^4 cfu/mL	12/16[(c)]
Accuracy		100% (20 out of 20) of the contaminant-only PT samples above the system LOD were positive.		
Specificity		96% (23 out of 24) of the unspiked interferent and DW samples were negative.		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples. One unspiked CA DW replicate returned an inconclusive result.[(d)]		
False negatives		Four false negative results were obtained from the analysis of samples spiked with levels of *F. tularensis* above the system LOD. Three spiked CA DW and one spiked 0.5 mg/L each humic and fulvic acid replicates returned negative results.		
Precision		86% (18 out of 21) of the sample sets showed consistent results among the individual replicates within that set.[(b, c, d)]		

[(a)] Infective/lethal dose.
[(b)] One spiked 0.5 mg/L each humic and fulvic acid replicate had one negative and three positive results.
[(c)] One spiked CA DW replicate had one positive and one negative results in the split samples. This indicated an inconclusive result and would require re-analysis in a real-world scenario. The remaining replicates were negative.
[(d)] One unspiked CA DW replicate had one positive and one negative results in the split samples. This indicated an inconclusive result and would require re-analysis in a real-world scenario. The remaining replicates were negative.

Table 7-3. *Y. pestis* Target 1 Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	0.28 cfu/mL[(a)]	0/4[(a)]
			2×10^3 cfu/mL	4/4
			5×10^3 cfu/mL	4/4
			1×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
	Interferent PT samples	Humic and fulvic acids	1×10^4 cfu/mL	24/24
	DW samples	Concentrated DW	1×10^4 cfu/mL	16/16
Accuracy		100% (16 out of 16) of the contaminant-only PT samples above the system LOD were positive.		
Specificity		100% (24 out of 24) of the unspiked interferent and DW samples were negative.		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples.		
False negatives		No false negative results were obtained from the analysis of the interferent and DW samples spiked with levels of *Y. pestis* above the system LOD.		
Precision		100% (21 out of 21) of the sample sets showed consistent results among the individual replicates within that set.		

[(a)] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *Y. pestis*.

Table 7-4. *Y. pestis* Target 2 Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	0.28 cfu/mL[(a)]	0/4 [(a)]
			2×10^3 cfu/mL	4/4
			5×10^3 cfu/mL	4/4
			1×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
	Interferent PT samples	Humic and fulvic acids	1×10^4 cfu/mL	24/24
	DW samples	Concentrated DW	1×10^4 cfu/mL	16/16
Accuracy		100% (16 out of 16) of the contaminant-only PT samples above the system LOD were positive.		
Specificity		100% (24 out of 24) of the unspiked interferent and DW samples were negative.		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples.		
False negatives		No false negative results were obtained from the analysis of the interferent and DW samples spiked with levels of *Y. pestis* above the system LOD.		
Precision		100% (21 out of 21) of the sample sets showed consistent results among the individual replicates within that set.		

[(a)] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *Y. pestis*.

Table 7-5. *B. anthracis* Target 1 Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	200 cfu/mL[a]	0/4[b]
			2×10^3 cfu/mL	3/4[c]
			5×10^3 cfu/mL	4/4
			1×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
	Interferent PT samples	Humic and fulvic acids	1×10^4 cfu/mL	24/24
	DW samples	Concentrated DW	1×10^4 cfu/mL	12/16[d, e]
Accuracy		94% (15 out of 16) of the contaminant-only PT samples above the system LOD were positive.		
Specificity		100% (24 out of 24) of the unspiked interferent and DW samples were negative.		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples.		
False negatives		Two false negative results were obtained from the analysis of samples spiked with levels of *B. anthracis* above the system LOD, one for spiked NY DW and the other for spiked CA DW. Inconclusive results were found for one replicate each for spiked CA DW, spiked FL DW, and DI water at 2×10^3 cfu/mL.[c, d, e]		
Precision		76% (16 out of 21) of the sample sets showed consistent results among the individual replicates within that set.[b, c, d, e]		

[a] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *B. anthracis*.

[b] Three samples in the infective/lethal dose PT sample replicates had one positive and one negative result in the split samples. This indicated an inconclusive result and would require re-analysis in a real-world scenario. The remaining replicate was negative.

[c] One PT sample replicate at 2×10^3 cfu/mL had one positive and one negative result in the split sample. This indicated an inconclusive result and would require re-analysis in a real-world scenario. The remaining replicates were positive.

[d] One spiked CA DW replicate had one positive and one negative result in the split sample. This indicated an inconclusive result and would require re-analysis in a real-world scenario. Two of the remaining replicates were positive, the other negative.

[e] One spiked FL DW replicate had one positive and one negative result in the split sample. This indicated an inconclusive result and would require re-analysis in a real-world scenario. Two of the remaining replicates were positive.

Table 7-6. *B. anthracis* Target 2 Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	200 cfu/mL[a]	0/4[b]
			2×10^3 cfu/mL	3/4
			5×10^3 cfu/mL	4/4
			1×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
	Interferent PT samples	Humic and fulvic acids	1×10^4 cfu/mL	24/24
	DW samples	Concentrated DW	1×10^4 cfu/mL	13/16[c]
Accuracy		94% (15 out of 16) of the contaminant-only PT samples above the system LOD were positive.		
Specificity		100% (24 out of 24) of the unspiked interferent and DW samples were negative.		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples.		
False negatives		Two false negative results were obtained from the analysis of samples spiked with levels of *B. anthracis* above the system LOD, one for spiked CA DW and the other for DI water at 2×10^3 cfu/mL. Inconclusive results were found for two spiked CA DW replicates.[c]		
Precision		86% (18 out of 21) of the sample sets showed consistent results among the individual replicates within that set.[b, c]		

[a] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *B. anthracis*.

[b] Two samples in the infective/lethal dose PT sample replicates had one positive and one negative result in the split samples. This indicated an inconclusive result and would require re-analysis in a real-world scenario. The remaining replicates were negative.

[c] Two spiked CA DW sample replicates had one positive and one negative result in the split sample. This indicated an inconclusive result and would require re-analysis in a real-world scenario. One remaining replicate was positive, the other negative.

Table 7-7. *B. anthracis* Target 3 Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	200 cfu/mL[a]	0/4[b]
			2×10^3 cfu/mL	4/4
			5×10^3 cfu/mL	4/4
			1×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
	Interferent PT samples	Humic and fulvic acids	1×10^4 cfu/mL	24/24
	DW samples	Concentrated DW	1×10^4 cfu/mL	12/16[c]
Accuracy		100% (16 out of 16) of the contaminant-only PT samples above the system LOD were positive.		
Specificity		100% (24 out of 24) of the unspiked interferent and DW samples were negative.		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples.		
False negatives		No false negative results were obtained from the analysis of samples spiked with levels of *B. anthracis* above the system LOD. All four replicates for spiked CA DW returned inconclusive results.[c]		
Precision		95% (20 out of 21) of the sample sets showed consistent results among the individual replicates within that set.[b]		

[a] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *B. anthracis*.

[b] Two samples in the infective/lethal dose PT sample replicates had one positive and one negative result in the split samples. This indicated an inconclusive result and would require re-analysis in a real-world scenario. The remaining replicates were negative.

[c] All four spiked CA DW sample replicates had one positive and one negative result in the split sample. This indicated an inconclusive results for all replicates and would require re-analysis in a real-world scenario.

Table 7-8. *Brucella suis* Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	40 cfu/mL[a]	0/4 [b]
			2×10^3 cfu/mL	2/4 [c]
			5×10^3 cfu/mL	4/4
			1×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
	Interferent PT samples	Humic and fulvic acids	1×10^4 cfu/mL	24/24
	DW samples	Concentrated DW	1×10^4 cfu/mL	16/16
Accuracy		88% (14 out of 16) of the contaminant-only PT samples above the system LOD were positive.		
Specificity		100% (24 out of 24) of the unspiked interferent and DW samples were negative.		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples.		
False negatives		No false negative results were obtained from the analysis of samples spiked with levels of *Brucella suis* above the system LOD.		
Precision		90% (19 out of 21) of the sample sets showed consistent results among the individual replicates within that set. [b, c]		

[a] Infective/lethal dose—below the R.A.P.I.D.® System LOD for *Brucella suis*.

[b] One sample in the infective/lethal dose PT sample replicates had one positive and one negative result in the split samples. This indicated an inconclusive result and would require re-analysis in a real-world scenario. The remaining replicates were negative.

[c] Two PT sample replicate at 2×10^3 cfu/mL had one positive and one negative result in the split sample. This indicated an inconclusive result and would require re-analysis in a real-world scenario. The remaining replicates were positive.

Table 7-9. *E. coli* Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	0.2 cfu/mL[a]	0/4
			2×10^3 cfu/mL	4/4
			5×10^3 cfu/mL	4/4
			1×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
	Interferent PT samples	Humic and fulvic acids	1×10^4 cfu/mL	20/20[b]
	DW samples	Concentrated DW	1×10^4 cfu/mL	16/16
Accuracy		100% (16 out of 16) of the contaminant-only PT samples above the system LOD were positive.		
Specificity		100% (24 out of 24) of the unspiked interferent and DW samples were negative.		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples.		
False negatives		No false negative results were obtained from the analysis of samples spiked with levels of *E. coli* above the system LOD.		
Precision		100% (20 out of 20) of the sample sets showed consistent results among the individual replicates within that set.		

[a] Infective/lethal dose— below the R.A.P.I.D.® System LOD for *E. coli*

[b] One set of spiked 0.5 mg/L each humic and fulvic acid replicates were suspected of having cross-contamination problems. The samples were not rerun, so the results are not reported here for consistency with other rerun reporting.

Other performance factors: A technician with prior PCR experience operated the R.A.P.I.D.® System at all times. All three components of the R.A.P.I.D.® System (the ITI 1-2-3 Flow Kit, the freeze-dried reagents, and the R.A.P.I.D.® 7200 instrument) were straightforward and easy to use. Three separate work areas were needed for testing to minimize cross-contamination. The freeze-dried reagents were color coded, contained all of the necessary components for PCR in one vial, and were reconstituted in the same vial, making PCR setup easy. Reagents for the DNA purification and PCR setup had room temperature storage requirements. The glass capillaries used on the R.A.P.I.D.® 7200 instrument were problematic when the rotors on the carousel were dirty, but posed no problems once the rotors were properly cleaned. The sample throughput for this verification test was 36 to 72 samples per day. Approximate operational times were 2 hours for DNA extraction/purification, 30 minutes for reconstituting the reagents and loading the capillaries for one carousel batch, and 30 minutes/carousel batch for PCR. The R.A.P.I.D.® software was easy to use, and additional software analysis tools other than those used in this test are available. The cost for the R.A.P.I.D.® System is around $8 per sample for the ITI 1-2-3 Flow Kit DNA purification step, $17 to test each split sample using the freeze-dried reagents, and approximately $55,000 for the R.A.P.I.D.® 7200 instrument itself (20 inches x14 inches x10 inches, 50 pounds). The R.A.P.I.D.® 7200 instrument can hold up to 32 capillaries (14 split samples, plus controls).

Chapter 8
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